UNIVERSIDADE FEDERAL DO ESPÍRITO SANTO
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JULIANA MACEDO DELARMELINA

Bidens pilosa L.: análises da composição química e atividades biológicas de diferentes populações e condições de cultivo

VITÓRIA

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Tese apresentada ao Programa de Pós-Graduação em Biologia Vegetal do Centro de Ciências Humanas e Naturais da Universidade Federal do Espírito Santo, como requisito para obtenção do título de Doutor em Biologia Vegetal.

Orientadora: Prof[a] Dr[a] Maria do Carmo Pimentel Batitucci.

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Apresentada em 22 de junho de 2017.

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Prof[a]. Dr[a]. Maria do Carmo P. Batitucci - UFES
Orientadora e Presidente da Comissão

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VITÓRIA

2017

Aos meus amores, Jefferson e nosso filho, Gabriel, dedico com carinho.

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“As pessoas que acreditam em nossa capacidade fazem mais do que apenas incentivar.
Elas criam para nós uma atmosfera que favorece nosso sucesso”.

John Spalding

RESUMO

A utilização de plantas com fins medicinais, para tratamento, cura e prevenção de doenças, é uma das mais antigas formas de prática medicinal da humanidade. *Bidens pilosa* L., Asteraceae, popularmente como picão-preto, é uma planta tradicionalmente utilizada para o tratamento de hepatite, câncer, diabetes, entre outras desordens. É uma planta de interesse terapêutico por ser rica em compostos químicos associados à saúde humana. No entanto, os compostos químicos podem variar, qualitativamente e quantitativamente, de acordo com inúmeros fatores que podem, consequentemente, refletir em suas atividades biológicas. Dentre os principais fatores que contribuem para a variação da composição química de extratos vegetais tem-se a variabiliadade genética, fatores ambientais e o processo de extração, como a utilização de solventes com polaridade distintas, por exemplo. Assim, o presente estudo visou: a) avaliar a variabilidade do extrato hidroalcoólico e cinco de frações (hexano, diclorometano, acetato de etila, butanol e aquosa) de quatro populações de *B. pilosa* (Afonso Claudio, Barra de São Francisco, Cariacica e Muniz Freire) usando marcadores genéticos (RAPD), análises fitoquímicas (prospecção fitoquímica, ESI(-) FT-ICR MS e conteúdo total de flavonoides, taninos e compostos fenólicos), análises de atividade antioxidante *in vitro* (por meio dos ensaios de $DPPH^{\bullet}$, $ABTS^{\bullet+}$, atividade quelante sobre o Fe^{+2} e sistema β-caroteno/ácido linoleico) e, análises *in vivo* para avaliar a citotoxicidade, anticitotoxicidade, mutagenicidade e antimutagenicidade (pelo teste do micronúcleo em medula óssea de camundongos). b) avaliar a variabilidade do extrato da planta submetida a três condições de cultivo (orgânico, inorgânico e controle) e em diferentes estágios fenológicos (vegetativo e floração), por meio de análises fitoquímicas, antioxidantes e *in vivo* (mutagenicidade e citotoxicidade das plantas no estágio de floração) e de crescimento. As análises fitoquímicas revelaram significativa diferença quantitativa e qualitativa entre as amostras testadas, o que refletiu em variabilidade nas atividades biológicas (antioxidante, t-test, $P<0.5$). Os resultados sugerem que os fatores ambientais foram determinantes, em comparação aos fatores genéticos. Os ensaios *in vivo* demonstraram que a planta não induziu citotoxicidade e mutagenicidade em todas as condições experimentais (Tukey, $P<0.5$) e foi capaz de proteger o DNA contra os danos induzidos pela ciclofosfamida, nos ensaios de anticitotoxicidade e antimutagenicidade.

Palavras-chave: Análise fitoquímica • atividade antioxidante • *Bidens pilosa* L. • ESI(-) FT-ICR MS • fertilizante • micronúcleo.

ABSTRACT

The use of plants for medicinal purposes, for treatment, cure and prevention of diseases, is one of the oldest forms of medicinal practice of mankind. Bidens pilosa L., Asteraceae, popularly called "picão-preto", is a plant traditionally used for the treatment of hepatitis, cancer, diabetes, among other disorders. It is a plant of therapeutic interest because it is rich in chemical compounds associated with human health. However, chemical compounds can vary, qualitatively and quantitatively, according to numerous factors that reflect on their biological activities. Among the main factors that contribute to the chemical variation of plant extracts is the genetic variability, environmental factors and the extraction process, such as the use of solvents with different polarity, for example. Thus, the present study aimed to: a) evaluate the variability of the hydroalcoholic extract and five fractions (hexane, dichloromethane, ethyl acetate, butanol and aqueous), from four populations of B. pilosa (Afonso Claudio, Barra de São Francisco, Cariacica and Muniz Freire), using genetic markers (RAPD), phytochemical analyzes (phytochemical prospecting, ESI (-) FT-ICR MS and total content of flavonoids, tannins and phenolics), analyzes of antioxidant activity *in vitro* (by $DPPH^{\bullet}$, $ABTS^{\bullet+}$,, chelating activity on Fe^{+2} and β-carotene/linoleic acid assays), and *in vivo* analyzes to evaluate cytotoxicity, anti-cytotoxicity, mutagenicity and antimutagenicity (by micronucleus test in mouse bone marrow). b) evaluate the variability of hydroalcoholic extract of plants submitted to three growth conditions (organic and inorganic fertilizers and the control) and in different phenological stages (vegetative and flowering), through phytochemical, antioxidant and in vivo analyzes (mutagenicity and cytotoxicity of plants in the flowering stage) and growth. The phytochemical analyzes revealed a significant quantitative and qualitative difference between the samples tested, which reflected in variability in their biological activities (antioxidant, t-test, $P<0.5$). The results suggest that environmental factors were determinant as compared to genetic factors. *In vivo* assays demonstrated that the plant did not induce cytotoxicity and mutagenicity in all experimental conditions (Tukey, $P<0.5$) and was able to protect DNA from damage induced by cyclophosphamide in the anti-cytotoxicity and antimutagenicity assays.

Keywords: Antioxidant activity • *Bidens pilosa* L. • ESI (-) FT-ICR MS • fertilizers • micronucleus • phytochemical analysis.

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Abs_1	absorbância da amostra
Abs_o	absorbância do controle
ABTS	2,2'-azino-bis(3-etilbenzotiazolina-6-ácido sulfônico)
$ABTS^{\circ+}$	ABTS radicalar
CAT	catalase
CPA	ciclofosfamida
DNA	ácido desoxirribonucléico (do inglês *Deoxyribonucleic acid*)
DPPH	2,2-difenil-1-picril-hidrazila
DPPH-H	difenil-picril-hidrazina
GpX	glutationa peroxidase
GSH	glutationa
H_2O_2	peróxido de hidrogênio
HNE	4-hidroxi-2,3-nonenal
HOO°	radical hidroperoxil
INCA	Instituto Nacional de Câncer
MDA	malondialdeído
MNPCE	eritrócito policromático micronucleado (do inglês *micronucleated polychromatic erythrocytes*)
N^7	nitrogênio 7
NCE	eritrócito normocromático (do inglês *normochromatic erythrocytes*)
$O_2^{\circ-}$	ânion superóxido
OH°	radical hidroxila
OMS	Organização Mundial de Saúde
PAL	fenilalanina amonialiase

PCE	eritrócito policromático (do inglês *polychromatic erythrocytes*)
RNS	espécies reativas de nitrogênio (do inglês *Reactive nitrogen species*)
$ROO^{\cdot}$	radical peroxil
ROS	espécies reativas de oxigênio (do inglês *Reactive oxygen species*)
SOD	superóxido dismutase
UFES	Universidade Federal do Espírito Santo

SUMÁRIO

1 INTRODUÇÃO

1.1 Estresse oxidativo, doenças humanas e defesa antioxidante

O estresse oxidativo representa uma perturbação no estado de equilíbrio das reações pró-oxidantes e antioxidantes nos sistemas biológicos. Essa condição ocorre quando há uma superprodução de espécies reativas de oxigênio (*Reactive oxygen species*, ROS) e/ou nitrogênio (*Reactive nitrogen species*, RNS) e a deficiência de antioxidantes enzimáticos e não enzimáticos (VALKO et al., 2007).

As ROS e RNS são produtos altamente instáveis e reativos resultantes do metabolismo celular normal e de fatores ambientais. As espécies reativas derivadas de oxigênio representam a classe mais importante de espécies radicais geradas em sistemas vivos. A maioria das reações de produção de ROS envolve a redução parcial do oxigênio molecular, em reações que ocorrem naturalmente nos organismos aeróbios (MILLER; BUETTNER; AUST, 1990; SCHIEBER; NAVDEEP, 2014; ADEGOKE; FORBES, 2014; DORIS, 2015).

ROS é um termo coletivo para denominar moléculas, radicalares (radicais livres) ou não radicalares, derivadas do oxigênio (SHARMA et al., 2012). Os radicais livres contém um ou mais elétrons não emparelhados, o que lhes confere a elevada reatividade. Tais radicais são produzidos a partir da perda ou ganho de um elétron de uma molécula não-radical ou partir da quebra de uma ligação covalente (DORIS, 2015). Dentre as principais ROS produzidas pelo organismo podemos citar: ânion superóxido ($O_{2^{\cdot}}^{-}$), radical hidroxila ($OH^{\cdot}$), peróxido de hidrogênio (H_2O_2), radical peroxil ($ROO^{\cdot}$) e radical hidroperoxil ($HOO^{\cdot}$) (FINKEL; HOLBROOK, 2000).

1.1.1 Principais espécies reativas geradas no metabolismo celular

Há várias vias de formação das ROS endógenas. A produção de ânions superóxido ($O_{2^{\cdot}}^{-}$) ocorre principalmente nas mitocôndrias através da redução parcial do oxigênio molecular (adição de um elétron). Tal processo é mediado pela NAD(P)H oxidase e ocorre principalmente nos complexos I e III da cadeia transportadora de elétrons (VALKO et al., 2007; RIBEIRO et al., 2005; FINKEL; HOLBROOK, 2000).

Em solução aquosa, o $O_2^{\cdot -}$ pode ser convertido em peróxido de hidrogênio (H_2O_2) a partir da redução parcial do oxigênio molecular por dois elétrons (RIBEIRO et al., 2005). Essa conversão poderá ocorrer enzimaticamente, através da ação da superóxido dismutase (SODs) (em uma reação de dismutação), xantina oxidase, aminoácido oxidase e NAD(P)H oxidase. O H_2O_2 difunde-se facilmente pelas membranas celulares (BIRBEN et al., 2012; VASCONCELOS et al., 2007) e poderá gerar o radical hidroxila ($OH^{\cdot}$) na presença de metais de transição, como o Fe^{+2}, Fe^{+3} ou o Cu^{+2} (FENTON, 1984 *apud* BIRBEN et al., 2012).

O radical $OH^{\cdot}$ é produzido a partir de uma sucessão de reações denominadas reações de Haber-Weiss e Fenton (Figura 1) (BIRBEN et al., 2012). É o radical que possui maior potencial reativo e lesivo e, devido ao seu tempo de meia vida curto, dificilmente poderá ser neutralizado pelos mecanismos de defesa do organismo (HALLIWEEL; GUTERIDGE, 2015; VASCONCELOS et al., 2007; LONE et al., 2013). Há duas vias de controle da presença dos radicais $OH^{\cdot}$: o reparo dos danos causados por ele ou a inibição de sua formação (BARREIROS; DAVID, 2006).

$$Fe^{+3} + O_2^{\cdot -} \rightarrow Fe^{+2} + O_2 \qquad \textbf{Haber-Weiss}$$

$$Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + OH^- + OH^{\cdot} \qquad \textbf{Reação de Fenton}$$

Figura 1: Reações de Haber-Weiss e Fenton catalisadas por metais de transição (Fe^{+2}/Fe^{+3}). Adaptado de: Birben et al., (2012).

Muitos agentes oxidantes são capazes de elevar os níveis de ferro sérico por meio da redução da biossíntese de ferritina ou mesmo pelo aumento da biossíntese de receptores transferrina. Liochev e Fridovich (1994) demonstraram que o radical superóxido é capaz de libertar íons Fe^{+2}, por exemplo. Tais efeitos poderiam intensificar a ocorrência das reações de Fenton e aumentar a produção do radical hidroxila, altamente reativo (VALKO et al., 2007).

As espécies reativas, como radical $OH^{\cdot}$, podem causar danos a muitas biomoléculas e estruturas celulares (VALKO et al., 2007) por diferentes mecanismos, dentre eles as reações em cadeia de peroxidação lipídica. Esse radical pode abstrair um elétron de ácidos graxos poli-insaturados resultando em radical lipídico. O radical lipídico, por sua vez, reage com o

oxigênio produzindo radical peroxila (ROO•). Caso o radical ROO• não seja reduzido por antioxidantes, inicia-se uma reação em cadeia e há a transformação de ácidos graxos insaturados em hidroperóxidos. Os hidroperóxidos lipídicos são muito instáveis e se decompõem facilmente em produtos secundários, tais como aldeídos (4-hidroxi-2,3-nonenal - HNE, por exemplo) e malondialdeído (MDA) (BIRBEN et al., 2012). O MDA é um produto mutagênico e carcinogênico, enquanto o HNE parece ser o principal produto tóxico da peroxidação lipídica (VALKO et al., 2007). A tabela 1 apresenta as principais reações de produção de algumas espécies reativas de oxigênio.

Tabela 1: Espécie reativa de oxigênio e suas principais reações de produção. Adaptado de: Birben et al. (2012).

Oxidante	**Fórmula**	**Equação de reação**
Ânion Superóxido	$O_2^{\cdot -}$	$NADPH + 2O_2 \leftrightarrow NADP^+ + O_2^{\cdot -} + H^+$
		$2O_2^{\cdot -} + H^+ \rightarrow O_2 + H_2O_2$
Peróxido de hidrogênio	H_2O_2	Hipoxantina + H_2O_2 + $O_2 \leftrightarrow$ xantina + H_2O_2
		Xantina + H_2O + $O_2 \leftrightarrow$ ácido úrico + H_2O_2
Radical hidroxila	$OH^{\cdot}$	$Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + OH^- + OH^{\cdot}$
Radical peroxila	$ROO^{\cdot}$	$R^{\cdot} + O_2 \rightarrow ROO^{\cdot}$
Radical hidroperoxil	$HOO^{\cdot}$	$O_2^- + H_2O \leftrightarrow HOO^{\cdot} + OH^-$
Ácido hipocloroso	HOCl	$H_2O_2 + Cl^- \rightarrow HOCl + H_2O$

1.1.2 Papel das espécies reativas no organismo

As ROS apresentam, dependendo de sua concentração, um duplo papel nos organismos vivos. Em baixos níveis e em condições normais, o balanço redox controla funções fisiológicas relacionadas à resposta imune, inflamações, morte celular programada (apoptose), indução e manutenção de vias de transdução de sinais envolvidos no crescimento e diferenciação celulares, entre outras. No entanto, em elevadas concentrações, as ROS podem resultar em danos a lipídios, proteínas, açúcares e/ou ácidos nucleicos, inibindo o funcionamento normal dessas moléculas (VALKO et al., 2007; SCHIEBER; CHANDEL, 2014).

Dessa forma, o estresse oxidativo está associado a diversas condições patológicas humanas, dentre elas: doenças cardiovasculares, como aterosclerose e hipertensão, desordens neurológicas, como o Parkinson e Alzheimer, desordens oftalmológicas, diabetes *mellitus*, isquemia e injúria da reperfusão, câncer, entre outras (SHARMA et al., 2012; VALKO et al.,

2006; VASCONCELOS et al., 2007; FINKEL; HOLBROOK, 2000). Além disso, o processo de envelhecimento ocorre em grande parte devido à ação prejudicial das ROS. Alguns estudos demonstram a relação diretamente proporcional da idade com o aumento da peroxidação lipídica, da oxidação de proteínas e de danos ao DNA (HARMAN, 1956 *apud* VALKO et al., 2007).

Além da produção endógena de ROS, como relatado anteriormente, estamos diariamente expostos a diversos agentes oxidantes exógenos, que podem levar ao estresse oxidativo e contribuir para o desenvolvimento de diversas doenças e para o envelhecimento precoce. Dentre os principais agentes exógenos podemos destacar o tabagismo, o consumo excessivo de bebidas alcoólicas, a exposição ao ozônio, a exposição à radiação ultravioleta, a hiperoxia, a radiação ionizante, a exposição a metais pesados (tais como ferro, cobre e cádmo), a alimentação e a ingestão/inalação/aplicação de xenobióticos (BIRBEN et al., 2012; ADEGOKE; FORBES, 2014).

A formação de espécies reativas durante o metabolismo de xenobióticos é um importante mecanismo empregado por agentes tóxicos capazes de causar danos celulares e ao DNA (KUMARASAMY et al., 2002). A modificação permanente do DNA, resultante da ação de ROS, representa o primeiro passo na mutagênese, carcinogênese e envelhecimento (SHARMA et al., 2012; VALKO et al., 2006).

1.1.3 Estresse oxidativo *versus* mutagênese, carcinogênese e quimioterapia

O estresse oxidativo tem relação direta com várias doenças humanas bem como o processo de envelhecimento. O delicado equilíbrio entre os efeitos benéficos e maléficos das ROS é um aspecto importante para os organismos vivos e é alcançado por mecanismos de regulação redox (VALKO et al., 2007).

Algumas pesquisas demonstram que as ROS podem induzir, por diferentes mecanismos, danos ao DNA por meio de quebras, modificações nas bases púricas, pirimídicas e no açúcar (desoxirribose) e até mesmo realizar ligações cruzadas (MARNETT, 2000; VALKO et al., 2006; VASCONCELOS et al., 2007), resultando em danos irreversíveis (mutações).

Muitos fatores estão associados com o aumento do estresse oxidativo em pacientes com câncer e que fazem quimioterapia (VALKO et al., 2007), dentre eles estão incluídos a

liberação de íons de ferro (Fe^{+2} e Fe^{+3}) durante o tratamento (GARÓFOLO, 2003). Há cada vez mais evidências de que o desequilíbrio redox está relacionado à estimulação oncogênica e desempenha um importante papel em vários estágios da carcinogênese, visto que muitas pesquisas demonstram a elevada frequência de lesões oxidativas ao DNA em diversos tipos tumorais (VALKO et al., 2006; VALKO et al., 2007).

Além disso, a ação de drogas quimioterápicas utilizadas no tratamento antitumoral pode ser mediada pelo acúmulo intracelular de ROS e metais de transição (FAINTUCH et al., 1995, apud GARÓFOLO, 2003). A maioria dos quimioterápicos atua de forma inespecífica, sendo capazes de interferir, por meio de diferentes mecanismos, em funções bioquímicas vitais para a célula tumoral ou não tumoral (INCA 2008a). Dessa forma, os antineoplásicos podem induzir efeitos colaterais genotóxicos, mutagênicos e citotóxicos em células normais, o que poderia resultar na formação tumores secundários (INCA, 2008a; MANZI; KAO, 2008) e na ocorrência de efeitos colaterais diversos. Tal toxicidade é refletida principalmente em tecidos normais de rápida proliferação celular, como a medula óssea e o folículo capilar, por exemplo (INCA, 2008a; KADAM et al., 2007).

De fato, as mutações, espontâneas ou induzidas, estão envolvidas na gênese de doenças relacionadas a desordens genéticas, como o câncer (BHATTACHARYA, 2011; DeFLORA et al., 1996; WATERS et al., 1996). O acúmulo de mutações que geram as neoplasias ocorre principalmente em genes que desempenham um papel fundamental no desenvolvimento tumoral, como os oncogenes e os genes de supressão tumoral (INCA, 2008a, TSAO et al., 2004; RUDDON, 2007; MOREIRA et al., 2004; COOPER, 1995; DeFLORA, 1998). Dessa forma, a mutagênese tem um papel especial na iniciação da carcinogênese (BUNKOVA et al., 2005).

O desenvolvimento tumoral é um processo multifásico que envolve alterações endógenas (genéticas, hormonais, imunes e fisiopatológicas) e epigenéticas (BRASILEIRO FILHO, 2004; SORIA et al., 2003; COOPER, 1995). Os agentes externos, químicos, físicos ou biológicos, otimizam o processo de carcinogênese, uma vez que são potenciais geradores de espécies reativas e causadores de danos ao DNA (IARC, 2011).

O câncer é a segunda causa de mortalidade por doença no mundo, ficando atrás apenas das mortes causadas por doenças cardiovasculares (DELFINO, 2006; INCA, 2016). Atualmente, 20 milhões de pessoas no mundo são diagnosticadas com a doença e estimativas para o ano de

2016, válidas também para o ano de 2017, apontam para a ocorrência de aproximadamente 600.000 novos casos, por ano, no Brasil, dentre os quais, 291.090 ocorrerão somente na região sudeste, reforçando a magnitude do problema do câncer no país (INCA, 2016).

Assim, o câncer configura-se em um problema de saúde pública e econômico mundial, devido à necessidade de pesquisas e tratamentos de elevada complexidade e de alto custo (INCA, 2011). Nesse cenário, é fundamental que os esforços estejam direcionados para a orientação das estratégias de prevenção, controle e tratamento do câncer, com o objetivo de reduzir a incidência e a mortalidade por câncer, no Brasil e no Mundo.

1.1.3.1 Antineoplásicos alquilantes: ciclofosfamida

Os diferentes quimioterápicos antioneoplásicos existentes são classificados de acordo com seu mecanismo de atuação. Dentre as várias classes de antineoplásicos temos, por exemplo, os agentes hormonais, antimetabólicos, antimitóticos e alquilantes (ALMEIDA et al., 2005; INCA, 2008a; KADAM et al., 2007).

Os agentes alquilantes são quimioterápicos que atuam em todas as fases do ciclo celular e somente nas células que se encontram em proliferação, apresentando assim um mecanismo de ação ciclo-específico (INCA, 2017). Tais agentes são capazes de reagir com importantes componentes da molécula de DNA de diferentes maneiras: substituindo átomos de hidrogênio por radical alquil, formando ligações covalentes por reação de alquilação com qualquer molécula carregada negativamente e estabelecendo ligações cruzadas, de três formas diferentes (Figura 2), podendo até mesmo impedir a separação da dupla fita, durante o processo de replicação. Dessa forma, o DNA é o principal alvo dos agentes alquilantes, podendo acarretar lesões em células cancerígenas ou normais (ALMEIDA et al., 2005; COLVIN; HAIT, 2009; KADAM et al., 2007).

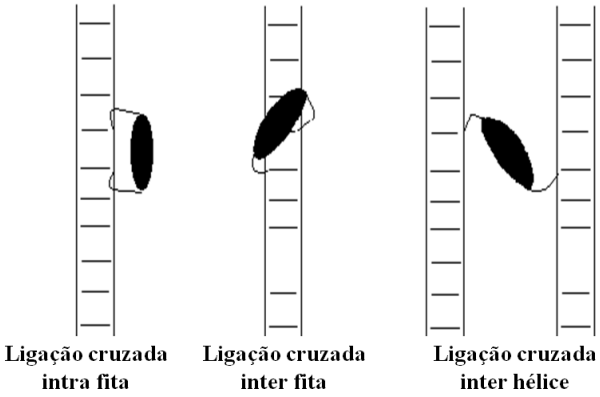


Figura 2: Algumas ligações cruzadas que os agentes alquilantes podem estabelecer com a molécula de DNA. Fonte: Almeida et al. (2005).

A ciclofosfamida (CPA, Figura 3) é um agente alquilante bifuncional, do tipo mostarda nitrogenada, que necessita de passar pelo processo de metabolização hepática, via isoenzimas do citocromo P-450, para adquirir sua atividade farmacológica, citotóxica e altamente mutagênica, que atuará nas células tumorais e, por vezes, nas não tumorais (COLVIN; HAIT, 2009; SALMON; SARTORELLI, 1995). Sua principal forma de atuação é via alquilação da base nitrogenada guanina (N^7) da molécula de DNA, o que pode acarretar: pareamento anormal com a timina (codificação errônea); quebra no anel imidazol da guanina; ligações cruzadas com as fitas de DNA; quebra das fitas do DNA devido a depurinação (KADAM et al., 2007; SALMON; SARTORELLI, 1995). Tais danos ao material genético poderão até mesmo ser observados microscopicamente.

Além disso, a CPA é um dos quimioterápicos que causam maior elevação dos níveis de ferro sérico que, como citado anteriormente, pode atuar como potente oxidante aumentando a produção de espécies reativas, principalmente OH° via reação de Fenton (Figura 1) (GARÓFOLO, 2003).

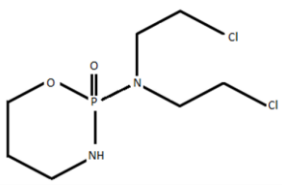


Figura 3: Estrutura química do agente alquilante ciclofosfamida (CPA).

Apresenta ampla aplicabilidade terapêutica sendo utilizada para o tratamento de muitos tipos de câncer como o de mama, leucemia, linfomas, entre outros (COLVIN; HAIT, 2009; LANCE et al., 2009; GENTILE et al., 1998). No entanto, sua utilização também é caracterizada por uma série de efeitos adversos, dentre os quais destacam-se cistite hemorrágica, leucopenia, supressão da medula óssea, enjoo e alopecia (LANCE et al., 2009; MANZI; KAO, 2008).

É comum observar pacientes fazendo o uso de uma terapia alternativa (complementação alimentar, uso de vitaminas, entre outros) para minimizar e/ou combater os efeitos fisiológicos adversos do tratamento quimioterápico. Muitos desses complementos terapêuticos são ricos em substâncias antioxidantes que podem atuar por diferentes mecanismos para diminuir os efeitos colaterais e promover a proteção das células "não-alvo" contra efeitos genotóxicos e mutagênicos induzidos (GENTILE et al., 1998; MITSCHER et al., 1996).

1.2 Defesas Antioxidantes

Algumas alterações no DNA causadas pela toxicidade das espécies reativas podem ser evitadas e/ou removidas por meio de mecanismos específicos e não específicos, como sistemas enzimáticos endógenos e a utilização de antioxidantes na dieta. Dessa forma, os mecanismos de defesa contra o estresse oxidativo e, consequentemente, contra a ocorrência de mutações induzidas por espécies reativas, podem envolver: a prevenção da formação de EROs e as defesas físicas e antioxidantes que eliminem as espécies formadas ou até mesmo elimine o excesso de metais de transição disponíveis (FILHO et al., 2011; VALKO et al., 2007; RIBEIRO et al., 2005).

Antioxidante pode ser definido como qualquer substância que, presente em baixa concentração, quando comparados a um substrato oxidável, atrasa ou inibe a oxidação desse substrato de maneira eficaz. Seu mecanismo de ação pode ser variável, podendo atuar na neutralização do radical e/ou inibição da oxidação por meio de doação/captura de um elétron, doação de átomos de hidrogênio, quelando metais de transição (Figura 4) e/ou por ação enzimática (MORAIS et al., 2013). Os antioxidantes podem, dessa forma, prevenir, impedir e reduzir danos oxidativos às moléculas nos organismos vivos (KRISHNAVENI et al., 2013), sendo potenciais inibidores da mutagênese e carcinogênese (FERGUSON, 1994).

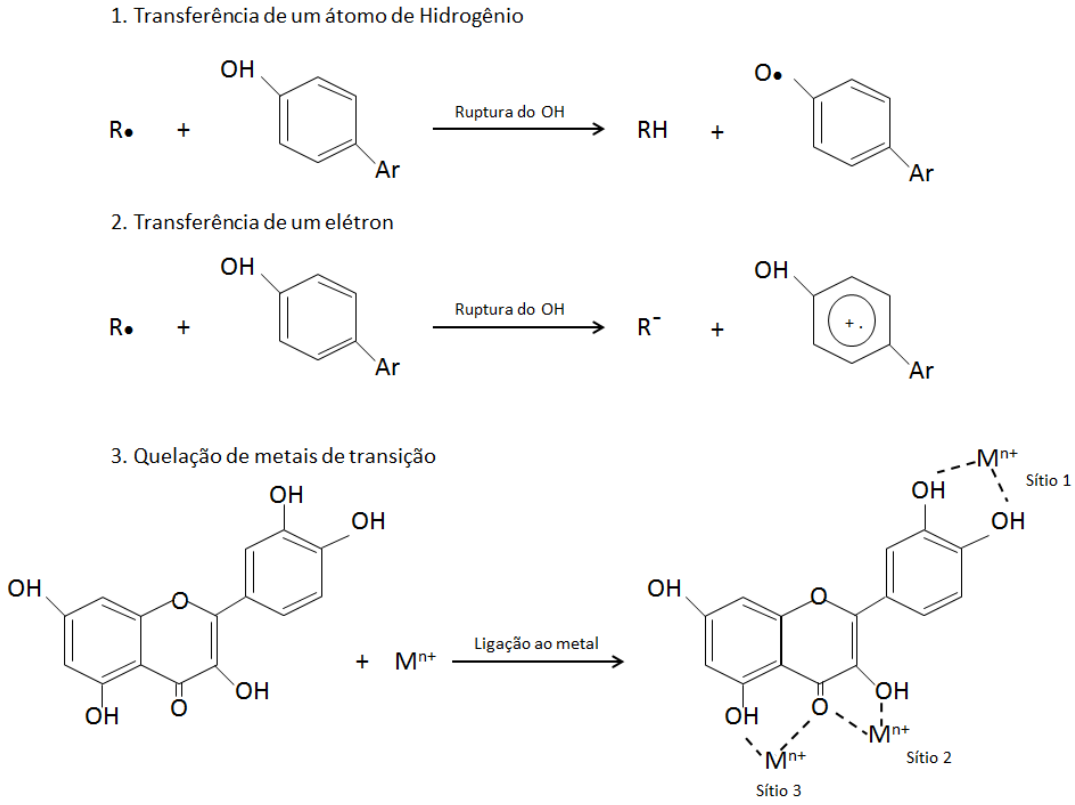


Figura 4: Possíveis mecanismos de ação de um agente antioxidante: transferência de um átomo de hidrogênio (1), doação ou captura de um elétron (2) e quelação de metais de transição, como o Fe^{+2} (3). Adaptado de Leopoldini, Russo e Toscano (2011).

As defesas antioxidantes enzimáticas são representadas, principalmente, pelas enzimas antioxidantes superóxido dismutase (SOD), glutationa peroxidase (GPx) e catalase (CAT). A SOD atua catalisando a dismutação do radical $O_2^{\bullet -}$ a H_2O_2 e O_2; a GPx atua sobre peróxidos em geral, com a utilização da glutationa (GSH) como cofator, convertendo-os a H_2O; e, a CAT, atua na decomposição do H_2O_2 a O_2 e H_2O (Figura 5) (VASCONCELOS et al., 2007; VALKO et al., 2007).

$$2\ O_2^{\bullet -} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

$$2\ GSH + H_2O_2 \xrightarrow{GPx} 2\ H_2O + GSSG$$

$$2\ H_2O_2 \xrightarrow{CAT} 2\ H_2O + O_2$$

Figura 5. Equações químicas das principais vias de atuação dos antioxidantes enzimáticos superóxido dismutase (SOD), glutationa peroxidase (GPx) e catalase (CAT). Adaptado de Vasconcelos e colaboradores (2007).

As defesas antioxidantes não-enzimáticas, por sua vez, incluem agentes encontrados naturalmente na dieta ou de forma sintética (medicamentos), capazes de reduzir a frequência de espécies reativa e metais de transição, com consequente redução de danos ao DNA (propriedades antimutagênicas e anticarcinogênicas) (HALLIWELL, 2007; FILHO et al., 2011; VALKO et al., 2007). São representadas pela GSH, ácido ascórbico, carotenoides, α-tocoferol, flavonoides, entre outras.

Dessa forma, a eliminação das espécies reativas é considerada a primeira linha de defesa contra o estresse oxidativo (HEINRICH; DHANJI; CASSELMAN, 2011) e, consequentemente, configura-se em uma boa estratégia para a prevenção e tratamento de muitas doenças degenerativas causadas por mutações. Diferentes fontes de antioxidantes são especialmente importantes para evitar, prevenir ou remover danos induzidos pelo estresse oxidativo, diretamente ou indiretamente (HALLIWELL, 2007a; 2007b; LANDETE, 2013). No entanto, devido aos possíveis efeitos adversos de antioxidantes sintéticos, que podem ter efeitos colaterais secundários, tais como a indução da carcinogênese (EBRAHIMABAD et al., 2010), as indústrias alimentícias e farmacêuticas têm voltado sua atenção para os antioxidantes naturais, que podem ser utilizados como um aditivo alimentar ou como suplemento farmacêutico (NICOLI et al., 1999; TLILI et al., 2014).

É sabido que várias plantas possuem significante propriedade antioxidante e diferentes classes de fitoquímicos estão associadas por serem responsáveis por essa atividade (KRISHNAVENI et al., 2013). No entanto, algumas substâncias presentes no extrato de plantas podem exibir tanto atividade antioxidante como pro-oxidante, dependendo da concentração e do sistema biológico em questão (PROCHÁZKOVÁET; BOUŠOVÁ; WILHELMOVÁ, 2011; DORMAN; HILTUNEN, 2011). Esse resultado é interessante quando a morte celular, por meio da necrose ou apoptose induzidas via estresse oxidativo, é um mecanismo desejado para a eliminação de células tumorais, por exemplo (DORMAN; HILTUNEN, 2011; ŠAMEC et al., 2014).

Muitas pesquisas demonstraram que vários compostos naturais, tais como compostos fenólicos, apresentam ampla atividade biológica, incluindo anticarcinogênica, antimutagênica, antioxidante e antimicrobiana (ATTIA, 2008; VALDEZ-MORALES et al., 2014; VALDÉS et al., 2015). Diante disso, a presença de compostos bioativos, como os antioxidantes, tem-se mostrado uma boa alternativa de proteção para o corpo humano contra os danos induzidos por espécies reativas (MORAIS et al., 2013).

1.2.1 Métodos para detecção da atividade antioxidante

Devido ao complexo processo de oxidação-antioxidação e a variedade de componentes antioxidantes, nenhum método isolado é capaz de fornecer um quadro abrangente do perfil antioxidante de uma determinada amostra (KHOUDJA; BOULEKBACHE-MAKHLOUF; MADANI, 2014; SWAPANA et al., 2013). Dessa forma, para avaliar o potencial antioxidante de uma substância é necessário conjugar diferentes metodologias, a fim de analisar seus possíveis mecanismos de atuação.

Vários métodos são utilizados para determinar a atividade antioxidante em extratos vegetais e substâncias isoladas. Dentre os mais usados tem-se: a avaliação da capacidade de sequestrar/neutralizar radicais livres, a avaliação da atividade quelante de íons Fe^{+2} e a avaliação da inibição da peroxidação lipídica a partir do sistema ácido linoleico/β-caroteno.

Radicais livres estão envolvidos na propagação de danos celulares. Dessa forma, antioxidantes com a capacidade de sequestrar radicais livres podem ter uma grande relevância na prevenção e tratamento de doenças induzidas por danos causados por eles (HASAN et al., 2009). Os ensaios com o radical livre estável difenil-picril-hidrazina (DPPH) e o 2,2'-azino-bis(3-etilbenzotiazolina-6-ácido sulfônico) (ABTS) são amplamente utilizados para avaliar substâncias redutoras e invetigar a atividade de eliminação de radicais livres. Ambos os testes são colorimétricos, facilmente reprodutíveis e de rápida execução (COTELLE et al., 1996; KHOUDJA; BOULEKBACHE-MAKHLOUF; MADANI, 2014).

O $DPPH^{\cdot}$ é um radical livre estável de cor violeta, obtido por dissolução do reagente em solvente orgânico, com absorbância máxima na faixa de 510-520nm (RUFINO et al., 2007). O ensaio é baseado no decréscimo da absorbância da solução contendo o radical na presença de um antioxidante doador de elétron ou hidrogênio (AH), devido à formação de uma forma não-radicalar DPPH-H (Figura 6) (SOUSA et al., 2007). Ao ocorrer a redução do $DPPH^{\cdot}$, a intensidade da cor da solução mininui tornando-se amarela. Dessa forma, a estabilização do radical ($DPPH^{\cdot}$) por um substrato leva a perda da cor púrpura e serve como um marcador (SZABO et al., 2007; GAIKWAD et al., 2010).

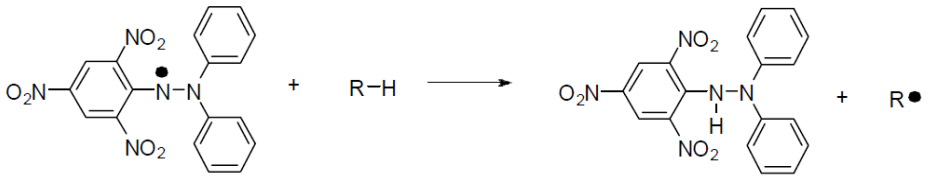


Figura 6: Estabilização do radical DPPH◦ por um substrato (R-H) que doa hidrogênio, resultando em um composto não-radicalar, DPPH-H. A coloração da solução na ausência de um antioxidante doador de elétrons ou hidrogênio é violeta; ao colocar a solução em contato com um antioxidante, a mesma torna-se amarela. Fonte: Szabo et al., 2007;

O método ABTS baseia-se na geração do radical ABTS ($ABTS^{\bullet+}$), por meio de uma reação química, eletroquímica ou enzimática. Normalmente, utiliza-se persulfato de potássio para geração do radical e realização do ensaio, adquirindo uma coloração escura, azul-esverdeada, após a reação (Figura 7). A presença de substâncias antioxidantes capazes de capturar/neutralizar o $ABTS^{\bullet+}$ causa o decréscimo da leitura da absorbância, que poderá ser mensurado após 6 minutos a 734nm. Sua solubilidade em solvente orgânico e inorgânico possibilita aferir a atividade antioxidante de compostos com natureza lipofílica ou hidrofílica (RUFINO et al., 2007). Além disso, muitas pesquisas demonstram que o ensaio ABTS é mais sensível na identificação da atividade antioxidante do que o ensaio DPPH, sendo isso atribuído à maior cinética de reação do ABTS e a menor capacidade do DPPH detectar a atividade antioxidante de compostos insolúveis em solventes orgânicos (com maior polaridade) (LEE et al., 2015).

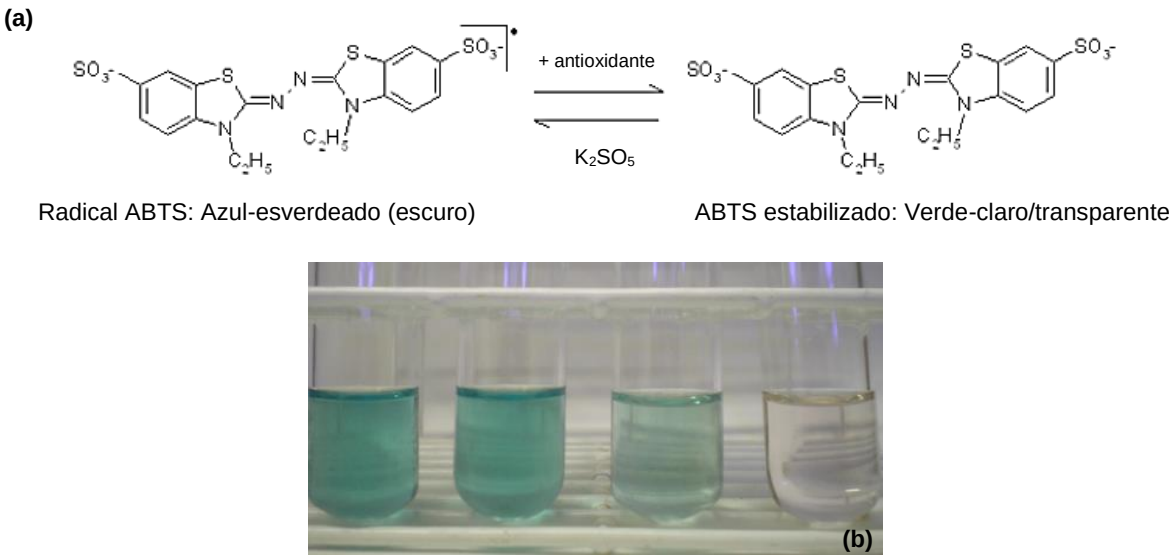


Figura 7. (a) Estabilização do radical ABTS●+ por um antioxidante e sua formação pelo persulfato de potássio. Ilustração de Edy de Souza de Brito adaptada de Rufino et al. (2007). Quanto maior a concentração de antioxidantes, maior a redução da absorbância (b).

A avaliação da atividade quelante é também amplamente utilizada para determinar a atividade antioxidante, uma vez que metais de transição, tais como o Fe^{+2} e Fe^{+3}, podem atuar como mediadores das reações de Haber-Weiss e Fenton, com consequente geração de espécies reativas e ocorrência de danos celulares (HALLIWEEL; GUTERIDGE, 2015; VASCONCELOS et al., 2007; LONE et al., 2013). Além disso, os metais podem reagir diretamente com moléculas, como os grupos tióis, para gerar radicais livres, ou ainda induzir vias de sinalização celulares (BIRBEN et al., 2012). Reid e colaboradores (1994) demonstraram que as ROS geradas por reações catalisadas por metais de transição podem causar substituições de bases nitrogenadas na molécula de DNA, principalmente a substituição de guanina por citosina.

A ferrozina (3-(2 piridil)- 5,6-difenil-1,2,4-triazina-4′,4″- ácido dissulfônico, sal sódico), utilizada amplamente para testes de atividade quelante, forma um complexo estável com o Fe^{+2}, resultando em uma solução de coloração roxo escuro (Figura 8). Na presença de um agente quelante, a formação do complexo estável é diminuída ou impedida, resultando em redução da intensidade da coloração, que é detectável em espectrofotômetro a 562 nm (TANG et al., 2002; BIRBEN et al., 2012).

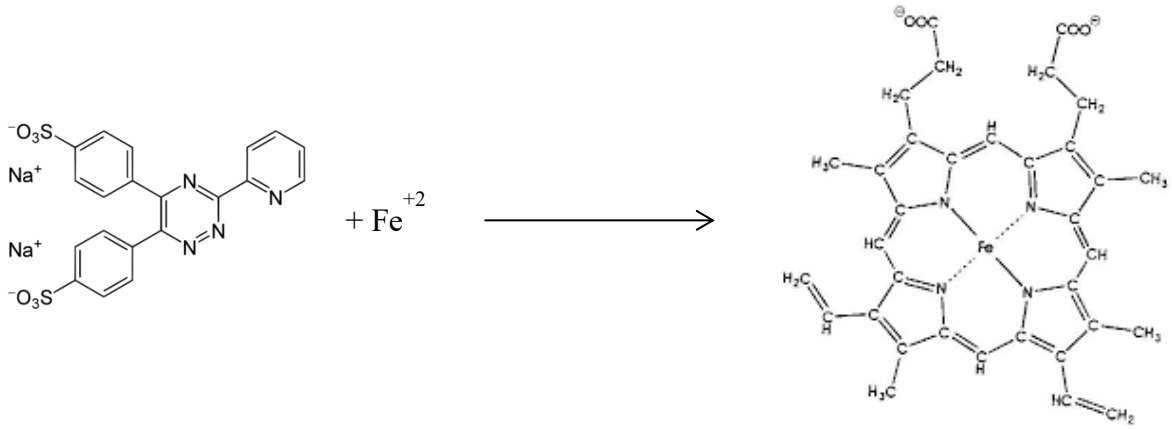


Figura 8. Formação do complexo estável da ferrozina com o Fe^{+2}

Os métodos de avaliação da diminuição e/ou inibição da peroxidação lipídica, por sua vez, avaliam a capacidade de um composto proteger moléculas de natureza lipídica, como as membranas celulares, por exemplo. O ensaio da inibição da co-oxidação do sistema β-caroteno/ácido linoleico tem como princípio a oxidação do ácido linoleico em elevada temperatura, com perda de um átomo de hidrogênio do carbono 11, entre duas ligações duplas (Figura 9). O radical formado irá atacar o β-caroteno, molécula altamente insaturada e que

apresenta coloração laranja, na tentativa de recuperar esse átomo de hidrogênio (RUFINO et al., 2006; CHIRINOS et al., 2013). Ao perder sua conjugação, os carotenoides perdem sua cor laranja característica (sofrem descoloração), sendo possível mensurar a redução da absorbância a 470nm. A taxa de perda da coloração (redução da absorbância) do β-caroteno é mais lenta na presença de substâncias antioxidantes capazes de neutralizar os radicais gerados durante a peroxidação do ácido linoleico (AMAROWICZ et al., 2004; LU; KNOO; WIART, 2014). Nesse ensaio nota-se o fenômeno denominado "paradoxo polar"; antioxidantes apolares exibem maiores atividades antioxidantes na emulsão, pois se concentram na fase lipídica. Já os antioxidantes polares, que dissolvem-se melhor na água, são menos efetivos na proteção de moléculas lipídicas (KOLEVA et al., 2002; RUFINO et al., 2006; MCCLEMENTS et al., 2000) .

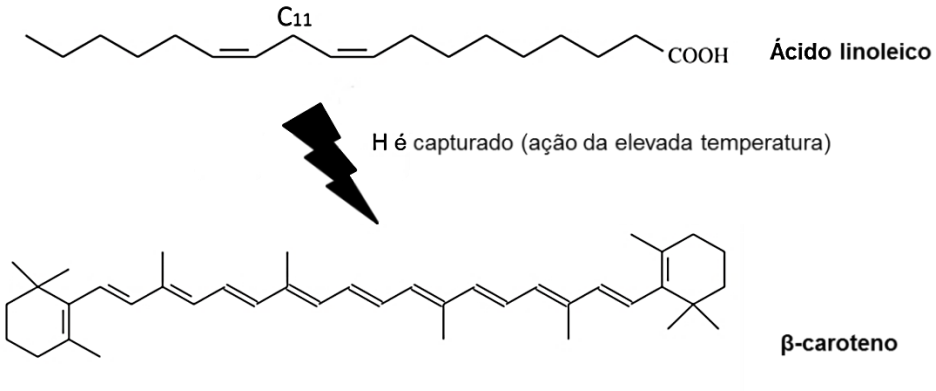


Figura 9. Inibição da co-oxidação do β-caroteno/ácido linoleico. O ácido linoleico, com a ação da temperatura elevada, sofre peroxidação (com a retirada do hidrogênio do carbono 11). Ao sofrer a oxidação, o ácido linoleico "ataca" a molécula de β-caroteno na tentativa de recuperar o átomo perdido. A co-oxidação do β-caroteno/ácido linoleico resulta no decréscimo da absorbância. A presença de uma substância antioxidante apolar é capaz de estabilizar o radical formado (ácido linoleico oxidado) e evitar a oxidação do β-caroteno.

1.3 Composição química vegetal

Os vegetais sintetizam um vasto leque de compostos orgânicos que são tradicionalmente classificados em metabólitos primários e secundários. Os metabólitos primários ocorrem em todos os vegetais e tem papeis essenciais associados à fotossíntese, respiração, crescimento e o desenvolvimento. Estão incluídos nessa classe os carboidratos, lipídios, proteínas, fitoesteroides e nucleotídeos. Os metabólitos secundários, por sua vez, são estruturalmente diversos e com distribuição restrita, podendo ser utilizados para estudos de quimiotaxonomia. Não possuem função direta no crescimento e desenvolvimento vegetal, no entanto apresentam

grande importância ecológica, principalmente na proteção das plantas contra herbivoria e infecção microbiana, na atração de polinizadores e animais dispersores de sementes (através da cor, odor e sabor), na competição (como agente alelopático), na proteção contra raios ultravioletas e na sinalização molecular. Além disso, os metabólitos secundários tem despertado grande interesse da indústria alimentícia e farmacêutica, por serem potenciais fontes de novas drogas naturais, antibióticos, inseticidas e herbicidas e devido a sua ampla aplicabilidade como corantes, fibras, colas, óleos, ceras, agentes aromatizantes, drogas e perfumes (CROZIER; JAGANATH; CLIFFORD, 2006; TAIZ; ZEIGER, 2013).

A maioria dos metabólitos secundários é produzida por vias derivadas do metabolismo primário (Figura 10). Existem três classes principais de metabólitos secundários: compostos fenólicos, produzidos principalmente via rota do ácido chiquímico e rota do ácido malônico; terpenos, produzidos principalmente via rota do ácido mevalônico; e compostos nitrogenados, produzidos a partir dos aminoácidos alifáticos e rota do ácido chiquímico (CROZIER; JAGANATH; CLIFFORD, 2006; TAIZ; ZEIGER, 2013).

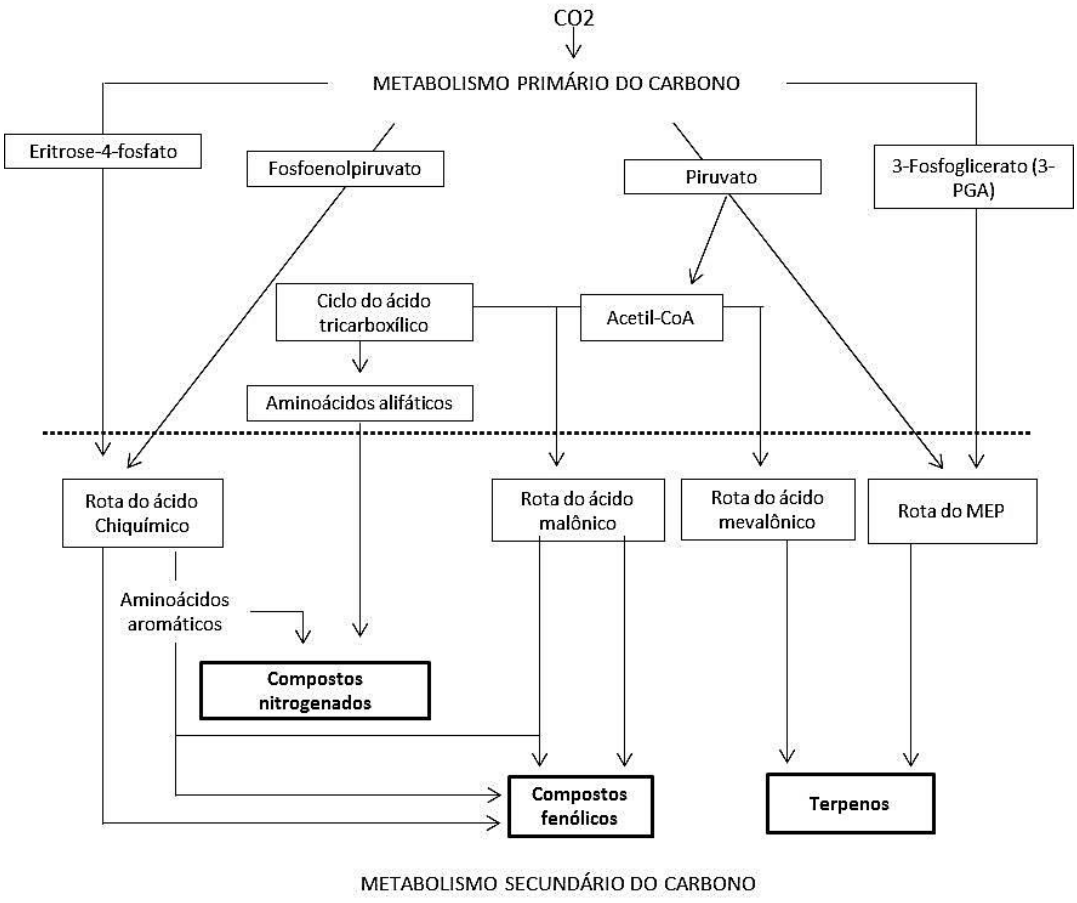


Figura 10: Principais vias de produção dos metabólitos secundários (compostos nitrogenados, compostos fenólicos e terpenos) e suas interligações. Adaptado de Taiz e Zeiger (2013).

1.3.1 Compostos fenólicos

Os compostos fenólicos possuem como pré-requisito pelo menos uma hidroxila (OH) ligada a um anel aromático (grupo fenol) (Figura 11). Sua classificação é usualmente baseada no número e no arranjo dos átomos de carbono além de serem comumente encontrados conjugados a moléculas de açúcares e ácidos orgânicos (CROZIER; JAGANATH; CLIFFORD, 2006; BALASUNDRAMA; SUNDRAMB; SAMMANA, 2006; TAIZ; ZEIGER, 2013). Estão incluídos nesse grupo os flavonoides, ácidos fenólicos, ligninas, taninos, entre outros (Tabela 2). Destes, a maior classe de compostos fenólicos é representada pelos flavonoides.

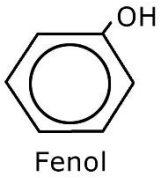


Figura 11: Estrutura química dos compostos fenólicos. Todo composto fenólico apresenta pelo menos uma hidroxila ligada a um anel aromático, constituindo assim um fenol.

Tabela 2. Classes de compostos fenólicos em plantas e sua estrutura carbônica básica.

Classe	Estrutura
Fenólicos simples, benzoquinonas	C_6
Ácidos hidroxibenzoicos	C_6–C_1
Acetofenonas, ácidos fenilacéticos	C_6–C_2
Ácidos hidroxicinâmicos, fenilpropanoides (cumarinas, isocumarinas)	C_6–C_3
Naftoquinonas	C_6–C_4
Xantonas	C_6–C_1–C_6
Estilbenos, antraquinonas	C_6–C_2–C_6
Flavonoides, isoflavonoides	C_6–C_3–C_6
Lignanas, neolignanas	$(C_6$–$C_3)_2$
Diflavonoides	$(C_6$–C_3–$C_6)_2$
Ligninas	$(C_6$–$C_3)n$
Taninos condensados (proantocianidinas ou flavolanos)	$(C_6$–C_3–$C_6)n$
Taninos hidrolisáveis	$(C_6$–$C_1)n$

Fonte: Adaptado de Balasundrama, Sundramb e Sammana (2006)

Os flavonoides possuem uma estrutura composta por 15 carbonos, sendo dois anéis aromáticos ligados por uma ponte de três carbonos (Figura 12). Os anéis possuem quantidades e posições variáveis de hidroxilas, que interferem na atividade antioxidante e hidrossolubilidade da molécula (LIEN et al., 1999; CROZIER; JAGANATH; CLIFFORD, 2006; TAIZ; ZEIGER, 2013).

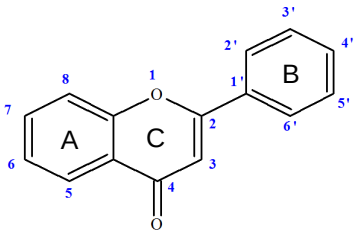


Figura 12: Estrutura química básica dos flavonoides composta por dois anéis aromáticos (A e B) ligados por uma ponte de três carbonos. O número e a posição das hidroxilas presentes no esqueleto carbônico variam de acordo com o tipo de flavonoide.

De fato, pesquisas demonstram que a estrutura química do flavonoide determina sua capacidade de atuar como antioxidante, principalmente via sequestro de radicais livres e atividade quelante de metais de transição. Tal atividade pode ser determinada por cinco fatores: reatividade como agente doador de hidrogênio e elétrons, estabilidade do radical flavanoil formado, reatividade frente a outros antioxidantes, capacidade de quelar metais de transição e solubilidade e interação com as membranas (LIEN et al., 1999; BARREIROS; DAVID; DAVID, 2006; GÓMEZ-RUIZ; LEAKE; AMES, 2007).

O grau de hidroxilação e metoxilação, o tipo de composto, a posição orto-di-hidroxila no anel B (grupo catecol, 3',4'-diidroxi) que favorece a estabilidade do radical livre flavanoil formado, a presença de OH nas posições 3', 4' e 5' e a presença de ligação dupla entre os C-2 e C-3 em conjugação com a função 4-oxo no anel C e/ou grupos OH nos carbonos 3 e 5, são essenciais para a atividade antioxidante dos flavonoides. Além disso, a dissociação das funções hidroxilas ocorre na sequencia 7-OH > 4’-OH > 5-OH (LIEN et al., 1999; RICE-EVANS; MILLER; PAGANGA, 1996; BALASUNDRAMA; SUNDRAMB; SAMMANA, 2006; LIEN et al., 1999; GÓMEZ-RUIZ; LEAKE; AMES, 2007).

Em geral, quanto menor o potencial de oxidação do flavonoide, maior é sua atividade sequestradora de radicais livres, e quanto maior o número de hidroxilas, maior a atividade como agente doador de H e de elétrons. Flavonoides monohidroxilados, como flavonas e flavononas, apresentam baixa atividade antioxidante (BARREIROS; DAVID; DAVID, 2006).

Variações nos padrões de substituição no anel C resultam nas principais classes de flavonoides, dentre elas flavonois, flavonas, flavononas, isoflavonas e antocianidinas (Figura 13). As substituições nos anéis A e B dão origem aos diferentes compostos dentro de cada classe de flavonoides. Essas substituições podem incluir oxigenação, alquilação, glicolisação, acilação e sulfatação (BALASUNDRAMA; SUNDRAMB; SAMMANA, 2006), que estão diretamente relacionados às diferentes atividades biológicas que tais compostos podem apresentar.

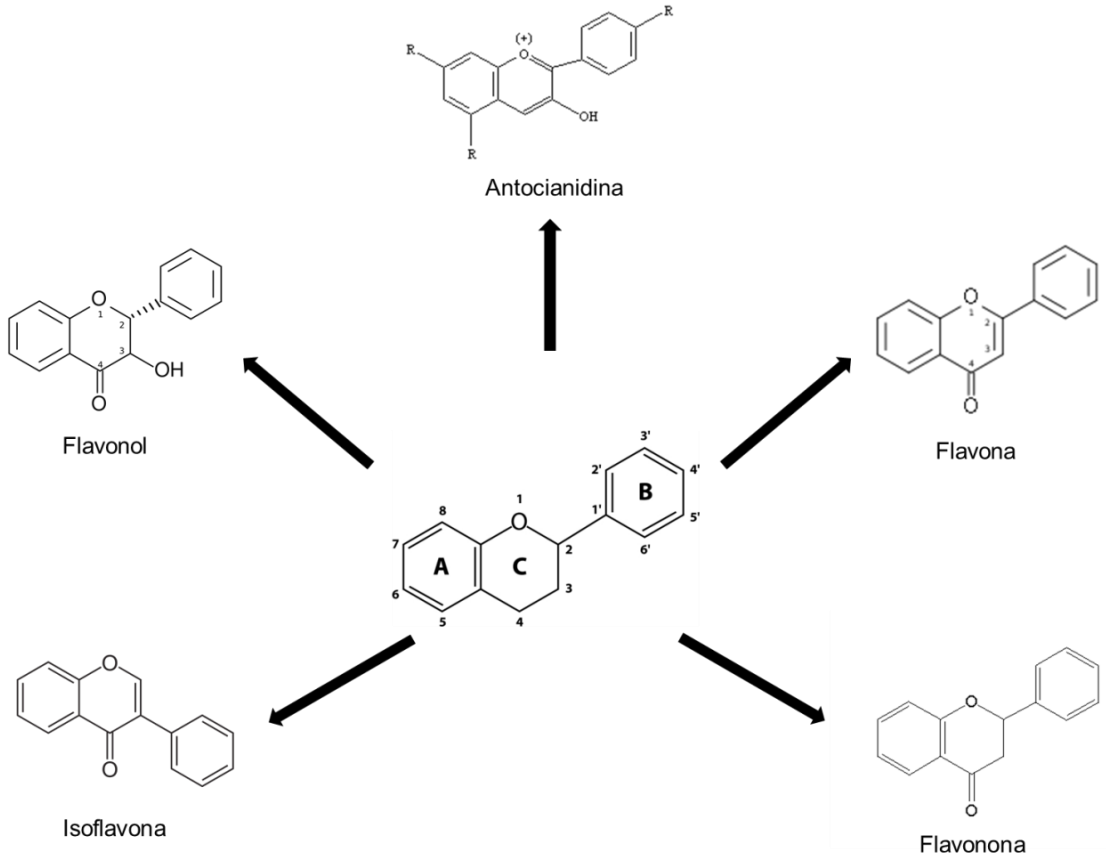


Figura 13: Estrutura genérica de algumas classes de flavonoides. Adaptado de Crozier; Jaganath e Clifford, 2006.

Muitos estudos reportam que os flavonoides apresentam importantes atividades biológicas, demonstrando-se efetivos como anticarcinogênicos, anti-inflamatórios, antioxidantes (YANG

et al., 2013; YAN et al., 2014; BREWER et al., 2014; PERICLEOUS et al., 2014), antivirais, anti-hipertensivos, anti-isquêmicos (PIRIE et al. 2014; SCHREUDER et al. 2014), anti-hiperglicêmicos, anticolesterolêmicos (TORREZAN et al. 2008), hepatoprotetor (DONG et al. 2013), além de apresentarem efeitos benéficos nos distúrbios da pós-menopausa (PERICLEOUS et al. 2014) e no retardando de doenças neurodegenerativas (XU et al. 2013; DAS et al., 2017).

No entanto, apesar de apresentarem ampla aplicabilidade biológica e agirem como bons agentes antioxidantes na forma reduzida, os compostos fenólicos podem apresentar efeitos nocivos ao organismo, com efeitos tóxicos em níveis sistêmico e celular, dependendo do tempo de exposição e das doses utilizadas (SILVA et al., 2015; VERMA et al., 2013). Compostos fenólicos, na presença de metais, como o Cu^{+2} e Fe^{+2}, formam um sistema metal-fenólico com produção de radical fenoxil, que pode exibir atividade pró-oxidante, com formação de radicais hidroxila (OH•), um dos principais responsáveis pela ocorrência de reações de quebra do DNA, por uma série de reações (Figura 14) (SAKIHAMA et al., 2002).

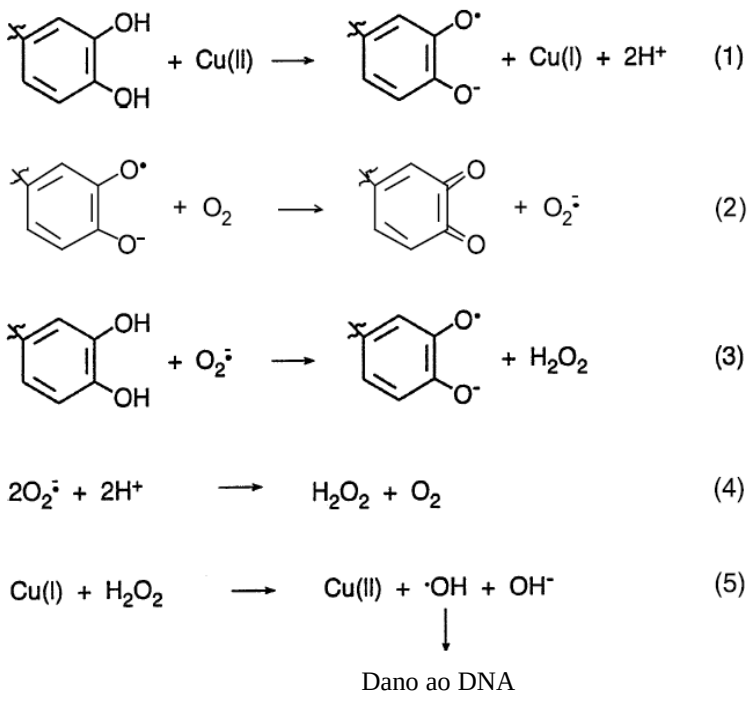


Figura 14: (1) Reação de oxidação do grupo catecol por Cu^{+2} gera semiquinona. (2) A semiquinona pode reagir com o oxigênio (O2) para formar o superóxido ($O2•^-$). (3) Essa reação é autocatalítica, uma vez que o $O2•^-$ pode oxidar o composto inicial para gerar semiquinona e peróxido de hidrogênio (H_2O_2). (4) O H_2O_2 também pode ser formado a partir do O2•. (5) Na presença de metal de transição, H_2O_2 pode ser rapidamente convertido em radical hidroxila (OH•) na reação de Fenton. Adaptado de Sakihama e colaboradores (2002).

1.3.2 Terpenos

Os terpenos constituem a maior classe de metabólitos secundários. Em geral, são compostos lipídicos insolúveis em água e que possuem uma estrutura básica com um número definido de unidades isoprênicas (C_5) (Figura 15). Sua classificação é realizada de acordo com o número de unidades C_5 que possui, como demonstrado na Tabela 3 (TAIZ e ZEIGER, 2013)

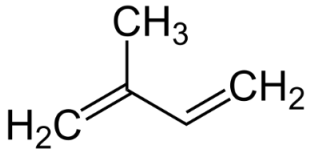


Figura 15: Unidade isoprênica (pentacarbonada). A unidade isoprênica é a unidade estrutural dos terpenos.

Tabela 3. Classificação dos terpenos pelo número de unidades isoprênicas (C_5), quantidade de átomos de carbono e exemplos. Tabela adaptada de Taiz e Zeiger (2013).

Terpenos	**Unidades C_5**	**Átomos de Carbono**	**Exemplos**
Monoterpenos	2	10	Ésteres piretroides
Sesquiterpenos	3	15	Ácido Abscísico
Diterpenos	4	20	Giberelina
Triterpenos	6	30	Esteroides/Brassinosteroides
Tetraterpenos	8	40	Carotenoides
Politerpenoides	>8	$[C_5]_n$	Dolicóis

Desempenham um importante papel biológico, principalmente ao nível de membranas celulares, crescimento e desenvolvimento vegetal. Grande parte dos terpenos são metabólitos relacionados com a função de defesa vegetal, inibindo a herbivoria devido sua propriedade inseticida e a capacidade de reduzir a palatabilidade. Ésteres monoterpenos, por exemplo, apresentam atividade inseticida. Monoterpenos e sesquiterpenos voláteis (óleos essenciais) conferem aroma característico com propriedades repelente e de advertência sobre a toxicidade do vegetal. As saponinas, composta de esteroides e triterpenos glicosídeos, são terpenos com

a capacidade detergente e emulsificante que atuam diretamente na defesa das plantas, contra herbívoros vertebrados (TAIZ e ZEIGER, 2013).

Além disso, têm-se alguns hormônios vegetais, tais como as giberelinas (diterpenos), brassinosteroides (triterpenos) e o ácido abscísico (sesquiterpenos), que atuam diretamente na regulação do metabolismo, crescimento e desenvolvimento vegetal; os caroteoides (tetraterpenos), que agem como pigmentos acessórios na fotossíntese e atuam contra a fotoxidação; os esteróis (triterpenos), que são componentes essenciais para a integridade das membranas celulares; entre outros (TAIZ e ZEIGER, 2013; LICHTENTHALER, 1999).

Alguns estudos demonstram que os terpenos possuem uma gama de atividades biológicas em animais, sendo capazes de proteger especialmente as membranas lipídicas dos danos induzidos pelo estresse oxidativo (COZZI et al., 1997; SOUZA et al., 2007). De fato, muitas pesquisas com extratos vegetais tem atribuído o potencial antioxidante da amostra ao teor de terpenos, em especial sesquiterpenos (óleos essenciais) e carotenoides (SOUZA et al., 2007; ANDRADE et al., 2013; TEIXEIRA et al., 2014; FERREIRA et al., 2014; RAŠKOVIĆ et al., 2014; SEPAHVANDA et al., 2014; PHAM et al., 2014;).

Muitas propriedades farmacológicas e terapêuticas dos terpenos já foram estudadas, demonstrando diversos efeitos, dentre eles: hepatoprotetor (RAŠKOVIĆ et al., 2014); anti-inflamatório (SOUZA et al., 2007); antibacteriano (SEPAHVANDA et al., 2014), antidiabético (MENDES, 2015), anticâncer, antimalárico, antituberculoso (EL SAYED et al., 2001; WANG; TANG; BIDIGARE, 2005), entre outros. Atualmente, algumas drogas baseadas em terpenos são comercializadas, tais como o quimioterápico Taxol® e o Artemisinin, utilizado para o tratamento da malária. Assim como ocorre nos compostos fenólicos, existe uma relação entre a estrutura molecular do terpeno com sua atividade biológica (WANG; TANG; BIDIGARE, 2005).

1.3.3 Compostos nitrogenados

Os compostos nitrogenados são metabólitos secundários que possuem nitrogênio como parte de sua estrutura. Incluem-se nessa categoria os alcaloides e os glicosídeos cianogênicos, compostos conhecidos por atuar na defesa das plantas contra a herbivoria e com considerável

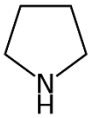
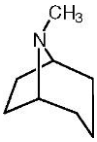
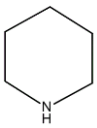
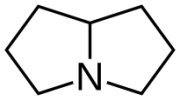
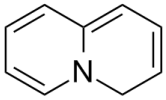
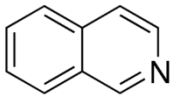
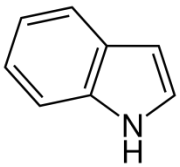
interesse devido ao seu potencial tóxico para humanos e às suas propriedades medicinais (TAIZ e ZEIGER, 2013).

Os alcaloides são conhecidos pelos importantes efeitos farmacológicos em animais vertebrados. Dentre as propriedades curativas que possuem incluem-se a acentuada ação no sistema nervoso, efeitos anti-hipertensivos, antiarrítmico, antimalárico, anticâncer, antibiótico, antimicrobiano, antidiabético, antioxidante, anestésico, tranquilizante, estimulante, tratamento de glaucoma, entre outros (ROBERTS; WINK, 1998; TAIZ; ZEIGER, 2013; TIONG et al., 2013; KHOSHIMOV ET AL., 2015).

Alguns alcaloides extraídos de plantas já são utilizados e comercializados para o tratamento de inúmeras doenças, tais como os quimioterápicos vincristina e vimblastina, que são utilizados no tratamento de leucemia linfoblástica aguda e diferentes linfomas, respectivamente, e a morfina, utilizada para aliviar dores intensas. Outros são compostos que causam dependência física e psíquica, tais como a nicotina (extraída das folhas de tabaco), atropina (antiespasmódico), cafeína (estimulante do sistema nervoso central), cocaína, eferidrina (broncodilatador e descongestionante), entre outros (Tabela 4). Além disso, alguns servem como modelo para a síntese de análogos com propriedades farmacológicas melhores (ROBERTS; WINK, 1998; TAIZ; ZEIGER, 2013).

Os glicosídios cianogênico, por sua vez, são compostos nitrogenados que liberam o ácido cianídrico (HCN), um gás tóxico de ação rápida, principalmente quando a planta é danificada por herbívoros, inibindo metaloproteínas fundamentais no processo de respiração celular. Alguns estudos demonstram seu efeito tóxico para animais (TAIZ; ZEIGER, 2013).

Tabela 4. Principais tipos de alcaloides, seus aminoácidos precursores, exemplos mais conhecidos e principais usos em humanos. Adaptado de Taiz e Zeiger (2013).

Classe de alcaloide	Estrutura	Precursos biossintético	Exemplos	Usos em humanos
Pirrolidínico		Ornitina (aspartato)	Nicotina	Estimulante, sedativo, tranquilizante
Tropânico		Ornitina	Atropina	Prevenção contra espasmos intestinais, antídoto contra outros venenos, dilatação de pupila para exame
			Cocaína	Estimulante do Sistema Nervoso Central, anestésico local
Piperidínico		Lisina (ou acetato)	Coniína	Veneno (paralisa os neurônios motores)
Pirrolizidínico		Ornitina	Retrorsina	Nenhum
Quinolizidínico		Lisina	Lupinina	Restabelecimento do ritmo cardíaco
Isoquinolínico		Tirosina	Codeína Morfina	Analgésico
Indólico		Triptofano	Psilocibina	Alucinógeno
			Reserpina	Tratamento de Hipertensão e psicoses
			Estricnina	Tratamento de distúrbios oculares

1.4 Fatores que influenciam a composição química vegetal

Nos últimos anos houve um aumento nas pesquisas sobre o papel de alguns metabólitos secundários como constituintes protetivos de algumas doenças na dieta. Ao contrário das vitaminas tradicionais que são essenciais para o bem-estar a curto-prazo, há evidências

crescentes de que a ingestão diária de baixas doses de metabólitos secundários, como os flavonoides, podem reduzir a incidência de cânceres e muitas doenças crônicas, incluindo doenças cardiovasculares e diabetes Tipo II (CROZIER; JAGANATH; CLIFFORD, 2006), além de ter aplicações práticas significativas em fins nutritivos e cosméticos e apresentar importância na adaptação das plantas ao estresse (RAMAKRISHNA; RAVISHANKAR, 2011).

No entanto, estudos demonstram que o teor de compostos químicos presentes em plantas pode variar de acordo com inúmeros fatores, tais como variabilidade genética, condições geográficas (altitude, por exemplo), temperatura, salinidade, disponibilidade hídrica, estresse biótico e/ou abiótico, luminosidade, composição do solo, entre outros, o que pode refletir diretamente em sua ação biológica terapêutica (SEIGLER, 1995; KÄHKONEN et al., 1999; LISIEWSKA; KMIECIK; KORUS, 2006; FRATIANNI et al., 2007; GOBBO-NETO; LOPES, 2007; FIGUEIREDO et al., 2008; KSOURI et al., 2008, CHIRINOS et al., 2013; BAIANO et al., 2013; TLILI et al., 2014). De fato, o acúmulo de metabólitos secundários ocorre com maior frequência em plantas sujeitas a tensões (RAMAKRISHNA; RAVISHANKAR 2011; SOUZA PINTO; KOLB, 2016).

Além disso, é sabido que diferentes partes da planta, como folhas, sementes, caules, flores e frutos, frequentemente diferem em sua composição química. Cada uma dessas partes pode variar seu teor químico nos diferentes estágios de desenvolvimento, nas diferentes épocas do ano ou até mesmo ao longo do dia (SEIGLER, 1995).

O desequilíbrio decorrente da exposição da planta a um fator biótico ou abiótico resulta em um estresse fisiológico e desencadeia uma série de efeitos metabólicos primários e secundários. Para melhor responder às alterações fisiológicas e fazer a manutenção do equilíbrio, os vegetais produzem os metabolitos secundários, dentre eles diversos grupos de antioxidantes, como mecanismo de proteção contra compostos oxidantes sintetizados diante das condições externas (TAIZ; ZAIGER, 2013; OH; TRICK; RAJASHIKAR, 2009). Dessa forma, a produção diferenciada de metabólitos secundários é um dos principais componentes de resistência aos fatores externos (BI; FELTON, 1995; SMIRNOFF, 1998; SINGH; AGRAWAL 2015).

Estudos *in vitro* e *in vivo* que relacionem os fatores ambientais com a biossíntese de metabólitos secundários têm sido utilizados para aumentar a produção ou induzir a síntese de

novos metabólitos de interesse. A variação da composição e concentração de vários produtos secundários de plantas são fortemente dependentes das condições de crescimento que, por diferentes mecanismos, podem alterar a expressão gênica das vias metabólicas responsáveis pela produção e acúmulo dos compostos relacionados (GOBBO-NETO; LOPES, 2007; RAMAKRISHNA; RAVISHANKAR, 2011).

Dessa forma, tendo em vista que há diversos fatores que influenciam e determinam o rendimento e a variabilidade química do metabolismo secundário de cada espécie, é importante saber como tais condições podem afetar a produção dos metabólitos, com o intuito de maximizar o rendimento de constituintes ativos com maiores benefícios nutricionais e para a saúde e minimizar os níveis de toxinas (FIGUEIREDO et al., 2008; ANDRÉ et al., 2009; SOUZA PINTO e KOLB, 2016).

Estudos demonstram que diversos fatores externos, tais como radiação UV-B, pré-tratamento com luz branca, deficiência nutricional e intensidade luminosa, são capazes de aumentar a expressão gênica de enzimas-chave para a produção de fenilpropanoides, por exemplo (LI et al., 1993; RAMAKRISHNA; RAVISHANKAR, 2011; LIU et al., 2006; ANDRÉ et al., 2009). Liu et al. (2006), correlacionaram a maior expressão de fenilalanina amonialiase (PAL), uma enzima considerada o primeiro passo-chave na via dos fenilpropanoides (Figura 16) e que catalisa a biossíntese de compostos secundários derivados da fenilalanina, com a maior produção do flavonoide quercetina. Li et al. (1993) demonstraram que a radiação UV-B induz a expressão gênica de PAL e chalcona sintase (CHS), enzima inicial na via dos flavonoides (LIU et al., 2006). Tal indução é fundamental para a biossíntese de metabólitos que reduzem os danos causados pela radiação.

Dessa forma, o passo de comprometimento na biossíntese de compostos secundários derivados da fenilalanina é catalisado pela fenilalanina amonialiase (PAL), enquanto a chalcona sintase (CHS) é a enzima crucial na via dos flavonoides, como demonstrado na Figura 16, passo 1 e 2, respectivamente (LI et al., 1993; LIU et al., 2006). Qualquer alteração induzida na expressão dessas enzimas-chave poderá alterar a biossíntese dos compostos relacionados.

André et al (2009), em estudos de expressão de 13 genes envolvidos na biossíntese de polifenois de cinco cultivares de batata, demonstraram que os diferentes perfis encontrados entre as cultivares estão correlacionados com variações na expressão gênica, quando os

tubérculos foram submetidos ao déficit hídrico. Os autores concluíram que a regulação da expressão gênica desempenha um papel essencial na produção de polifenois, além de ser distinta nos cinco cultivares estudados. Llorach et al. (2008) e Hassini et al. (2016) também demonstraram variação no conteúdo de fenóis totais de acordo com as variedades de alface e repolho, respectivamente, reportando que diferentes variedades podem influenciar no teor fitoquímico dos vegetais.

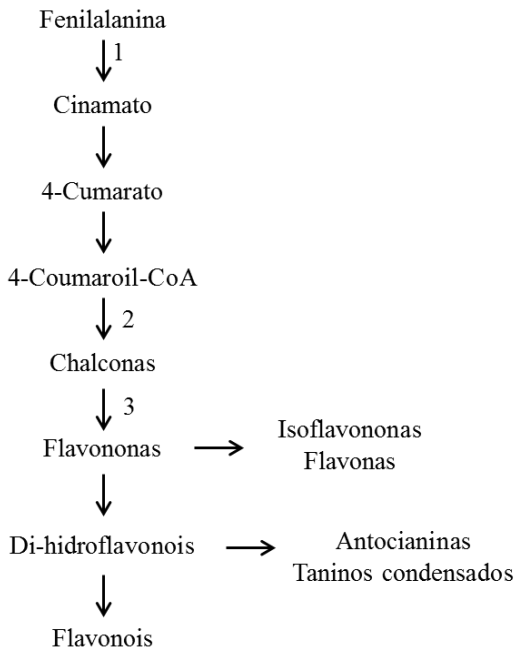


Figura 16: Vias para a biossíntese de produtos secundários derivados da fenilalanina em plantas. Adaptado de Taiz e Zeiger (2013) e Liu et al. (2006).

Singh e Agrawal (2015) demonstraram que o ritmo circadiano influencia no teor de metabólitos secundários, uma vez que proporciona o ajuste dos eventos fisiológicos de acordo com as mudanças que ocorrem ao longo do dia. Dessa forma, as respostas aos estresses ambientais previstos, podem ser mais adequadamente aliviadas e guiadas por ciclos luz/escuro e quente/frio, que estimulam diferentes vias metabólicas. Souza Pinto e Kolb (2016) demonstraram que a sazonalidade também pode ser fonte de variação de conteúdo para todas as classes de metabólitos secundários, ao estudar metabólitos fitotóxicos com finalidade herbicida. Segundo Gobbo-Neto e Lopes (2007), a época que um vegetal é coletado é um dos

fatores com maior importância, visto que a quantidade e/ou a natureza dos constituintes ativos não são constantes durante o ano.

Segundo Taiz e Zeiger (2013), os principais fatores abióticos que influenciam o crescimento e o desenvolvimento vegetal são os elementos minerais na solução do solo (fatores edáficos), a disponibilidade hídrica, a temperatura e a luz.

O estresse nutricional possui efeito marcante no nível de metabólitos secundários nos tecidos vegetais, especialmente compostos fenólicos. Estudos demonstram que a deficiência de nitrogênio (N), fosfato (PO_4), potássio (K), enxofre (S), ferro (Fe) e magnésio (Mg) podem aumentar a concentração de compostos fenólicos, em diferentes espécies de plantas (LEA et al., 2007; RAMAKRISHNA; RAVISHANKAR, 2011; SINGH; AGRAWAL, 2015). Outros estudos demonstram que solos pobres em nutrientes apresentam menor taxa de crescimento e maior biossíntese de todas as classes de metabólitos secundários, exceto para os compostos nitrogenados (GOBBO-NETO; LOPES, 2007).

A exposição à seca (déficit hídrico) ou ao estresse salino resulta em reações metabólicas semelhantes, uma vez que ambos os estresses levam a desidratação celular. Normalmente o déficit hídrico é acompanhado de elevadas temperatura e radiação UV, o que conduz a planta a um estado de estresse oxidativo, com consequente aumento no conteúdo de flavonoides e ácidos fenólicos. Além disso, reporta-se que a desidratação reduz o conteúdo de saponinas nos tecidos vegetais (GOBBO-NETO; LOPES, 2007; RAMAKRISHNA; RAVISHANKAR, 2011; AZHAR, 2011).

A luz é o fator abiótico fundamental para a ocorrência da fotossíntese, o principal processo metabólico das plantas. Embora seja essencial, a absorção da energia luminosa é uma fonte de espécies reativas de oxigênio, o que justifica a maior produção de metabólitos secundários com funções fotoprotetora e antioxidante, como alguns flavonoides e antocianinas, nas plantas submetidas à elevada condição de luminosidade (ROZEMA et al., 1997; SANCHEZ; SHIN; DAVIS 2011; RAMAKRISHNA; RAVISHANKAR, 2011). De fato, há uma correlação positiva entre a intensidade da radiação solar e a produção de compostos fenólicos (GOBBO-NETO; LOPES, 2007).

Estudos realizados com mutantes para a biossíntese de flavonoides demonstraram que as plantas geneticamente modificadas apresentam hipersensibilidade a radiação UV-B devido à ausência de acúmulo de flavonoides que absorvem a radiação, nas células da epiderme das

folhas. Dessa forma, o aumento da biossíntese de flavonoides induzida por UV-B é considerada uma resposta adaptativa a esse tipo de estresse. No entanto, embora a indução da expressão gênica por UV-B ter sido previamente demonstrada em uma variedade de espécies de plantas, os tipos de tecidos, o estágio de desenvolvimento, as condições de luz empregadas variam muito (LI et al., 1993).

Atualmente, poucas são as informações sobre o comportamento das plantas medicinais quando submetidas a diferentes condições de crescimento, mesmo sabendo que vários fatores podem afetar sua biomassa e produção de metabólitos (YUNES; CALIXTO, 2001; SIMÕES et al., 2013). No entanto, apesar de os estudos relacionados ao cultivo, manejo e produção de plantas medicinais não serem tão abrangentes e popularizados, como é o das plantas cultivadas, cada espécie medicinal, aromática ou condimentar, tem suas exigências e respondem de maneira diferenciada para diferentes níveis de agentes bióticos e abióticos (SOUZA et al., 2007), sendo necessários estudos mais aprofundados para o fornecimento de material vegetal bioativo de alta qualidade e com segurança para a indústria de fitoterápicos.

1.5 Mutagênese, antimutagênese e ensaios toxicológicos

Tem sido demonstrado que a mutagênese tem um papel especial na fase de iniciação da carcinogênese. Nela, a substância que atua sobre o DNA pode ser um agente, por si só, mutagênico ou pode ser um produto de um processo metabólico, que normalmente deveria ser inativado ou eliminado (BUNKOVA; MAROVA, 2005). É sabido que a ação mutagênica e carcinogênica de diversas substâncias envolve a promoção do estresse oxidativo celular, devido à geração de espécies reativas ao DNA (FERGUSON, 1994).

Os estudos de genotoxicidade, mutagenicidade e citotoxicidade constituem um passo importante na avaliação toxicológica dos medicamentos de origem vegetal onde podem estar presentes compostos mutagênicos relacionados com o desenvolvimento do câncer. Dentre os testes de avaliação de mutagenicidade e citotoxicidade preconizados pelas agências internacionais e instituições governamentais, o teste de micronúcleo em eritrócitos de medula óssea de roedores *in vivo* é amplamente aceito e recomendado para avaliação e o registro de novos produtos químicos e farmacêuticos que entram anualmente no mercado mundial (CHOY, 2001; RIBEIRO, et al., 2003).

O teste do micronúcleo em roedores visa detectar e quantificar a ação mutagênica e citotóxica de agentes indutores, físicos ou químicos (RABELLO-GAY, 1985; MACGREGOR, 1987), além de possibilitar a avaliação de agentes protetivos, com atividade antimutagênica e anticitotóxica. Em geral, esse bioteste permite deduzir que se o determinado agente está afetando o material genético do organismo teste, possivelmente apresentará efeitos semelhantes em qualquer tipo de célula, já que o código genético é universal (ALBERTS, 2004).

O teste *in vivo* é especialmente relevante uma vez que permite a consideração de fatores como a absorção, o metabolismo *in vivo*, a cinética do fármaco e o processo de reparo do DNA (KRISHNA, HAYASHI, 2000), o que dificilmente poderia ser reproduzido em ensaios *in vitro*. Além disso, esse ensaio apresenta algumas vantagens em relação aos outros, entre as quais podem ser citadas a elevada sensibilidade, o baixo custo, a confiabilidade e a rapidez na execução.

1.5.1 Teste do micronúcleo em medula óssea de roedores *in vivo*

O teste do micronúcleo pode ser executado em populações de células que estejam em constante divisão, sendo a medula óssea de mamíferos uma das regiões mais adequadas, visto que suas células levam de 22 a 24 horas para completar um ciclo celular e apresentam intensa atividade mitótica, o que as tornam suscetíveis aos danos genéticos (LUZHNA; KATHIRIA; KOVALCHUK, 2013; HEDDLE, 1973). Além disso, o teste é um dos métodos preferenciais utilizado pela comunidade científica, pois permite mensurar a perda de cromossomos inteiros e a ruptura cromossômica, resultante de eventos aneugênico e clastogênicos, respectivamente.

Dessa forma, o micronúcleo (MN) pode originar-se por quebra cromossômica (Figura 17b), que resulta em fragmentos cromossômicos acêntricos (evento clastogênico) ou devido às disfunções do fuso mitótico, durante o processo de divisão celular, em que cromossomos inteiros sofrem atraso em relação aos demais durante a migração para os polos da célula em anáfase (Figura 17a), não compondo o núcleo principal (evento aneugênico) (LUZHNA; KATHIRIA; KOVALCHUK, 2013; FENECH, et al., 2005). Eles apresentam até 1/3 do tamanho do núcleo principal, são redondos e não apresenta refringência.

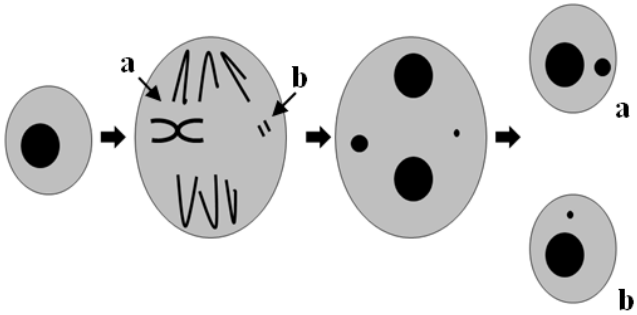


Figura 17: Diagrama ilustrativo demonstrando a origem do micronúcleo a partir de um evento aneugênico (a) ou de um evento clastogênico (b). Fonte: Adaptado de Fenech (2005).

O processo de eritropoiese (Figura 18) é fundamental para o entendimento do processo de formação do micronúcleo em células de medula óssea de camundongos. Durante a proliferação celular, uma substância teste administrada pode agir e causar quebras cromossômicas ou desordem nas moléculas que compõem as fibras do fuso mitótico, causando perda de cromossomos inteiros, refletindo, assim, na formação de um MN (KRISHINA; HAYASHI, 2000). Durante a maturação dos eritroblastos, o núcleo principal é expulso para transformar-se em eritrócito e, havendo MN, este permanece no citoplasma, onde é facilmente identificado por meio de análise citológica (HEDDLE, 1973).

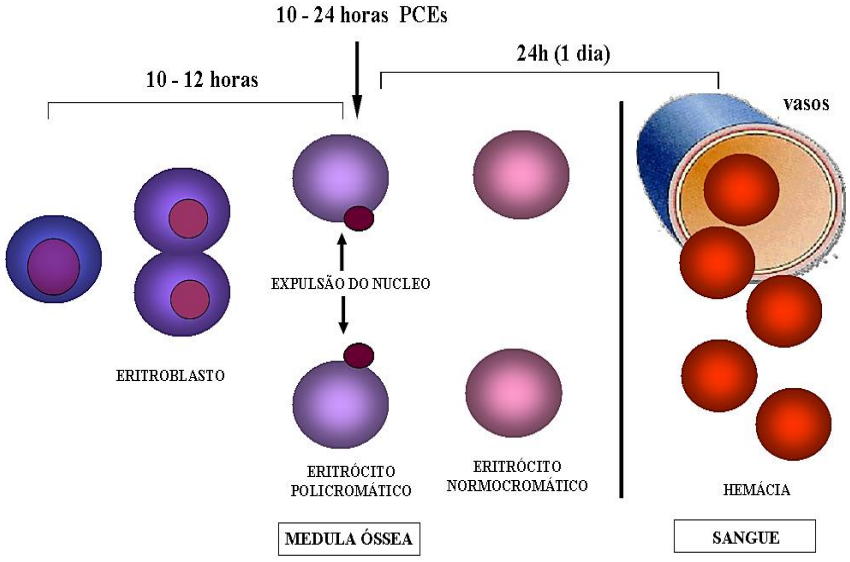


Figura 18: Processo de maturação dos eritrócitos que ocorre na medula óssea. Durante a maturação os eritrócitos policromáticos, que possui ribossomos e é considerado RNA positivo, perdem RNA passando a conter principalmente hemoglobina, tornando-se eritrócito normocromático (RNA negativo). Fonte: Adaptado de Ribeiro (2003).

A análise de micronúcleos é realizada apenas nas células imaturas, denominadas de eritrócitos policromáticos (PCE), que apresentam uma coloração azul quando em contato com eosina

azul de metileno seguimento Leishman, devido à presença de ribossomos e RNA em seu interior. Os eritrócitos permanecem imaturos (PCE) por um período de 10 a 24 horas e a análise de MN ocorre apenas nesse estágio de maturação, uma vez que haverá a garantia de que o dano ocorreu na mitose anterior, com a presença do agente teste. Posteriormente, os PCEs tornam-se maduros, passando a denominarem-se eritrócitos normocromáticos (NCE), que se coram em vermelho/rosa devido à presença de hemoglobina e a perda de RNA. Ao final do processo de eritropoiese, os NCEs irão para a corrente sanguínea, tornando-se hemácias (LUZHNA; KATHIRIA; KOVALCHUK, 2013; RIBEIRO, 2003; KRISHINA; HAYASHI, 2000).

Ao final do ensaio, é possível avaliar a frequência de eritrócitos policromáticos micronucleados (MNPCE) nos grupos experimentais, tanto nos testes de mutagenicidade quanto nos de antimutagenicidade. O aumento na frequência de MNPCE é um indicativo de que a substância é capaz de induzir danos ao DNA ou no aparato mitótico. Em contrapartida, quando uma substância é capaz de reduzir a frequência de MNPCEs frente aos danos induzidos por um mutágeno conhecido (quimioterápico antineoplásico, por exemplo), é indicativo de que essa substância pode atuar na proteção do DNA e do fuso mitótico, sendo, assim, considerada antimutagênica. Além disso, é possível avaliar a citotoxicidade a partir da relação de PCE em relação ao total de eritrócitos (PCE+NCE) (KRISHINA; HAYASHI, 2000). A redução da relação (PCE/(PCE+NCE) é indicativo de efeito citotóxico (RIBEIRO, 2003).

1.5.2 Atividade antimutagênica e prevenção de doenças

A prevenção de doenças relacionadas às mutações, como o câncer, pode ser alcançada de diversas maneiras, dentre elas evitando-se a exposição a agentes mutagênicos e reforçando-se os mecanismos de defesa endógeno, com o aumento da exposição a substâncias antimutagênicas, por exemplo (BHATTACHARYA, 2011; DeFLORA, 1998). Neste cenário, a busca de substâncias quimioprotetoras, com propriedades antimutagênicas e/ou anticitotóxicas, tem grande importância para a proteção da saúde humana (FERGUSON, 1994; HAYATSU et al., 1988; KNEŽEVIĆ-VUKČEVIĆ et al., 2005). De modo geral, todo agente antioxidante pode possuir atividade antimutagênica e anticarcinogênica (FERGUSON,

1994; MAMMADOV et al., 2009; BHATTACHARYA, 2011), apresentando assim, elevado potencial biológico.

Agentes antimutagênicos são compostos capazes de reduzir a frequência de mutações, espontâneas ou induzidas (LUZHNA; KATHIRIA; KOVALCHUK, 2013; VON BORSTEL; DRAKE; LOEB, 1996; GASIOROWSKI et al., 2001b). Tais substâncias podem atuar por diferentes mecanismos de ação dentre os quais podemos citar: prevenção da formação e a inativação de espécies reativas; ativação de mecanismos de desintoxicação de mutágenos; estímulo do reparo dos danos ao DNA; interferência no metabolismo de xenobióticos, entre outros (DeFLORA, 1998; FERGUSON, 1994; BUNKOVA et al., 2005; QARI, 2008). Independente do modo de ação, os antimutágenos podem ser classificados em dois grandes grupos: os que atuam por mecanismos de desmutagênese e os que atuam por bioantimutagênese (ANTUNES; ARAÚJO, 2000).

Na desmutagênese, os agentes antimutagênicos atuam na prevenção do dano causado pelo agente indutor (mutágeno), através da inativação, química ou enzimática, dos mutágenos (KADA; MORITA; INOUE, 1978; ANTUNES; ARAUJO, 2000). Agentes desmutagênicos são capazes de inativar os mutágenos antes que eles atuem sobre os genes (BHATTACHARYA, 2011; FERGUSON, 1994; OLIVEIRA et al., 2009). Na bioantimutagênese, por sua vez, os agentes atuam principalmente como moduladores do reparo e da replicação do DNA (KADA; MORITA; INOUE, 1978; ANTUNES; ARAUJO, 2000), sendo capazes de eliminar a mutação após a ação do mutágeno. Muitas substâncias antimutagênicas apresentam ambos mecanismos de ação (FERGUSON, 1994).

1.6 *Bidens pilosa L.*

Bidens pilosa L. conhecida popularmente como picão-preto, carrapicho e carrapicho-de-agulha, é uma planta daninha herbácea, ereta, pertencente à família *Asteraceae*, com porte entre 20-130 cm, originária da América do Sul e amplamente distribuída nas regiões tropicais e subtropicais do mundo, principalmente em áreas agrícolas (BARTOLOME; VILLASEÑOR; YANG, 2013; AMARAL et al., 2012; ABDOU et al., 2010). Apresenta folhas simples, geralmente tripartidas, pecioladas e opostas. Sua floração ocorre três vezes ao ano, com capítulos florais terminais e axilares, com flores marginais e centrais amarelas. Seu fruto é um aquênio linear de 5 a 9 mm de comprimento e provida de 2-3 aristas aderentes; sua

propagação é realizada via sementes, podendo chegar a 3.000 por planta (Figura 19) (LORENZI, 2000; AMARAL et al., 2012).

É considerada uma rica fonte alimentar e medicamentosa para humanos e outros animais, sendo seu cultivo promovido na África pela Food and Agricultural Organization devido algumas vantagens tais como seu rápido crescimento, ser comestível, palatável e apresentar uso seguro (BARTOLOME; VILLASEÑOR; YANG, 2013).

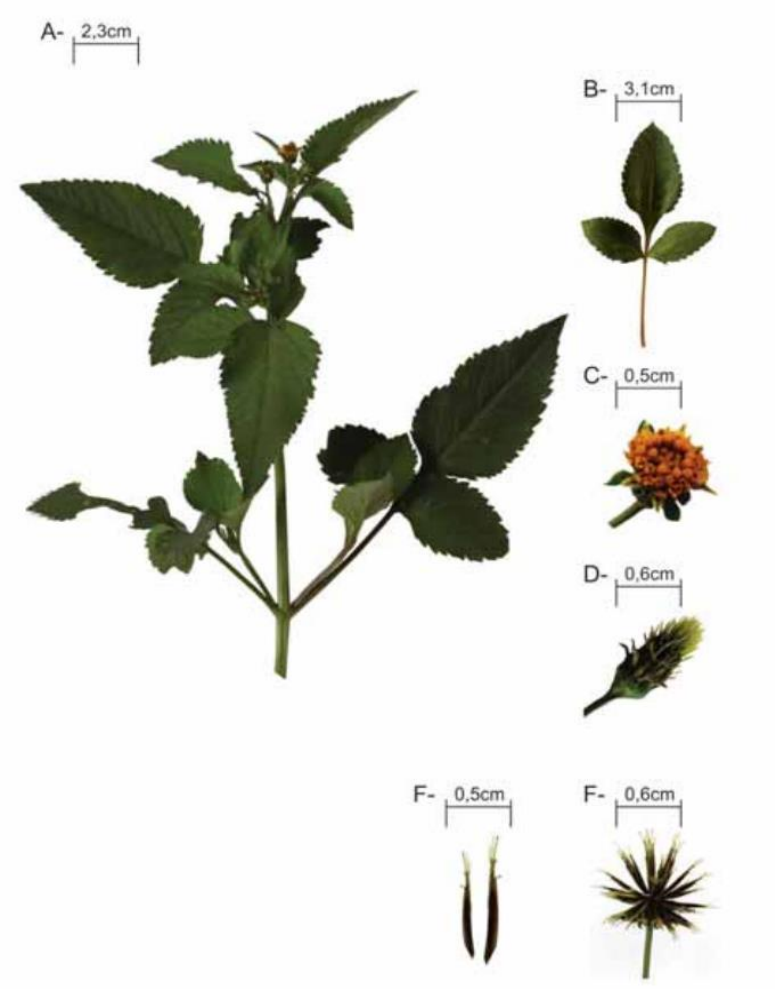


Figura 19: *Bidens pilosa* L. A- Aspecto geral do ramo florífero. B- Folha com margens serreadas. C- Flores amareladas reunidas em inflorescência tipo capítulo. D- Inflorescência. F- Frutos aquênios com ganchos aderentes na extremidade superior. Fonte: Amaral e colaboradores (2012).

Goza de notada importância pelo seu valor medicinal, sendo utilizada na medicina tradicional principalmente para o tratamento de inflamação, icterícia, alergia, diabetes, câncer, hepatite e muitas outras desordens (AMARAL et al., 2012; ARTHUR; NAIDOO; COOPOOSAMY, 2012; BARTOLOME; VILLASEÑOR; YANG, 2013). Muitos ensaios biológicos e

farmacológicos foram realizados, tendo sido reportado seus efeitos imunomodulatório (HORIUCHI; SEYAMA, 2008), anti-inflamatório (ALVAREZ et al., 1999; YOSHIDA et al., 2006; POZHARITSKAYA et al., 2010), anti-hiperglicêmico (HABECK, 2003), antimalárico (BRANDÃO et al., 1997; OLIVEIRA et al., 2004; KUMARI et al., 2009), antibacteriano (SILVA JUNIOR et al., 2014), hepatoprotetor (SUZIGAN et al., 2009), estrogênico (FRIDA et al., 2008), bem como citotoxicidade contra várias linhagens celulares (TAGAMI et al., 2009; ABDOU et al., 2010) incluindo células cancerígenas (SUNDARARAJAN et al., 2006; KVIECINSKI et al., 2008; KUMARI et al., 2009).

Kviecinski et al. (2008) demonstraram que o extrato hidroalcoólico de *Bidens pilosa* e frações obtidas a partir dos solventes acetato de etila e clorofórmio, foram efetivos na redução do tumor ascítico de Ehrlich em camundongos isogênicos Balb/c, quando administrados intraperitonealmente nas concentrações de 150 e 300mg/Kg. Estudos realizados pelos mesmos pesquisadores, por meio de análises de toxicidade mitocondrial e lisossomal, medidos através de ensaios de MTT e NRU *in vitro*, demonstraram que *Bidens pilosa* apresenta citotoxicidade sobre a linhagem celular Ehrlich.

Estudos *in vitro* realizados com cultura de células de hepatoma de ratos da espécie *Rattus norvegicus* (células HTC) demonstraram que o extrato hidroalcoólico, a infusão e a decocção de *B. pilosa* não apresentaram efeitos mutagênicos por meio do teste de micronúcleo *in vitro*. No entanto, a infusão foi capaz de induzir efeitos genotóxicos dose-dependente nas três concentrações utilizadas (10, 20 e 40 µL/mL), revelando que diferentes formas de preparo para a utilização da planta apresentam diferenças significativas em relação à indução da genotoxicidade. Dessa forma, tanto a dose utilizada como a forma de preparo sugerem precaução no uso fitoterápico dessa planta, que embora apresente muitos efeitos terapêuticos, não está livre de efeitos deletérios (COSTA et al., 2008).

Hong e colaboradores (2011) demonstraram que o extrato metanólico de *B. pilosa* não apresentou mutagenicidade em elevada concentração (5000 µg/placa) por meio do teste de Ames *in vitro* com as bactérias *Salmonella typhimurium* e *Escherechia* coli, na ausência e presença de ativação metabólica.

Experimentos realizados *in vivo*, com camundongos Swiss *Mus musculus*, demonstraram o efeito protetor do extrato glicolítico da planta contra a mucosite gastrointestinal induzida durante o tratamento quimioterápico/radioterápico, sendo capaz de atenuar as alterações

clínicas e patológicas com reestabelecimento da atividade proliferativa intestinal e proteção das células contra a morte celular, a partir de 100 mg/Kg. Tal efeito quimioprotetor tem sido atribuído as suas propriedades anti-inflamatórias (ÁVILA et al., 2015).

Ensaios de toxicidade aguda de folhas de *B. pilosa* em camundongos *in vivo* demonstraram o fraco efeito tóxico dos extratos etanólico e aquoso, com DL50 de 6,15 g/Kg e 12,30 g/Kg, respectivamente. Uma vez que as doses terapêuticas são mais baixas, sua utilização não demonstra riscos de toxicidade (FRIDA et al., 2008). Estudos de toxicidade em ratos Sprague Dawley tratados durante 28 dias com a infusão da planta, demonstraram a ausência de efeitos tóxicos associados ao tratamento, por meio de análises patológicas macro e microscópicas. Além disso, o tratamento prolongado com a planta aumentou a síntese de hemoglobina (CÁRDENAS et al., 2006).

Muitos autores reportaram a relação entre as atividades terapêuticas de *B. pilosa* e sua capacidade antioxidante e anti-inflamatória. De fato, muitos estudos demonstram que a parte aérea da planta apresenta capacidade de minimizar a peroxidação lipídica (ÁVILA et al., 2015; GOUDOUM et al., 2016), de atuar no sequestro/neutralização de radicais livres e de quelar metais de transição, evitando assim a formação de radicais hidroxila (KUSANO et al., 2003; CHIANG et al., 2004; MUCHUWETI et al., 2007; DEBA et al., 2008; YUAN et al., 2008). Além disso, Cortés-Rojas e colaboradores (2013) reportaram que as diferentes formas de preparo fitofarmacêutico da planta podem refletir em diferenças na atividade antioxidante. Dessa forma, seria adequado uma padronização para o controle de qualidade.

B. pilosa é uma fonte extraordinária de compostos químicos, particularmente flavonoides (SILVA et al., 2011; BARTOLOME et al., 2013). Apesar de possuir inúmeras propriedades medicinais, estudos que relacionem suas propriedades biológicas, tais como atividades antioxidante, citotóxica, mutagênica e antimutagênica, e seus teores de metabólitos secundários com a variabilidade genética, diferentes localizações de crescimento e diferentes condições de cultivo da planta, não foram realizados até o momento. Tal abordagem se faz necessária para que o uso dessa planta ofereça os benefícios de suas propriedades terapêuticas sem colocar em risco a saúde humana.

2. OBJETIVOS

2.1 Geral

Avaliar a influência da localização geográfica, da fertilização e do estágio fenológico na composição fitoquímica e nas atividades antioxidante, citotóxica, mutagênica e antimutagênica do extrato de *Bidens pilosa* L.

2.2 Específicos

No estudo com as plantas obtidas em diferentes localizações geográficas

- Avaliar as diferenças na composição química de extratos de *Bidens pilosa* L. obtidos a partir de plantas coletadas em quatro localidades do Espírito Santo.

- Avaliar o efeito mutagênico do extrato hidroalcoólico de *B. pilosa* nas concentrações de 100mg.kg^{-1}, 200mg.kg^{-1} e 300mg.kg^{-} por meio da análise da frequência de micronúcleos em células de medula óssea de camundongos albinos *Swiss* (*Mus musculus*) *in vivo*, para as quatro localidades avaliadas;

- Avaliar efeito antimutagênico do extrato hidroalcoólico de *B. pilosa* nas concentrações de 100mg.kg^{-1}, 200mg.kg^{-1} e 300mg.kg^{-}, através do protocolo de pré-tratamento, por meio da análise da frequência de micronúcleos em células de medula óssea de camundongos albinos *Swiss* (*M. musculus*) *in vivo*, para duas localidades avaliadas;

- Avaliar o efeito do extrato hidroalcoólico de *B. pilosa* (100mg.kg^{-1}, 200mg.kg^{-1} e 300mg.kg^{-}) na redução da frequência de micronúcleos induzidos pela ciclofosfamida por meio do cálculo da porcentagem de redução de danos, para duas localidades avaliadas;

- Avaliar se o extrato hidroalcoólico de *B. pilosa* (100mg.kg^{-1}, 200mg.kg^{-1} e 300mg.kg^{-}) induz citotoxicidade, por meio da relação entre o número de eritrócitos policromáticos e o número total de eritrócitos (eritrócitos policromáticos mais eritrócitos normocromáticos) em células de medula óssea de camundongos albinos *Swiss* (*M. musculus*) *in vivo*, para as quatro localidades avaliadas;

- Avaliar o efeito do extrato hidroalcoólico de *B. pilosa* (100mg.kg^{-1}, 200mg.kg^{-1} e 300mg.kg^{-}) contra à citotoxicidade induzida pela CPA no protocolo de pré-tratamento, por meio da relação entre o número de eritrócitos policromáticos e o número total de eritrócitos (eritrócitos policromáticos mais eritrócitos normocromáticos) em células de medula óssea de camundongos albinos *Swiss* (*M. musculus*) *in vivo*, para duas localidades avaliadas;

- Avaliar a capacidade antioxidante do extrato hidroalcoólico de *B. pilosa* e suas respectivas frações de polaridade crescente, obtidas a partir de plantas coletadas em quatro regiões do Espírito Santo, por meio dos testes de DPPH (2,2-difenil-1-picril-hidrazila); ABTS (2,2'-azino-bis(3-etilbenzotiazolina-6-ácido sulfônico), Sistema β-caroteno/ácido linoleico e atividade quelante dos íons Fe^{+2};

- Avaliar e comparar resultados obtidos, nos experimentos por meio de análises de correlação.

No estudo com as plantas obtidas no cultivo

- Avaliar a influência da fertilização e do estágio fenológico no crescimento e composição fitoquímica do extrato hidroalcoólico de *Bidens pilosa*;

- Avaliar a influência da fertilização e do estágio fenológico na capacidade antioxidante do extrato hidroalcoólico de *B. pilosa* obtido de plantas submetidas a três condições de cultivo, por meio dos testes de DPPH (2,2-difenil-1-picril-hidrazila); ABTS (2,2'-azino-bis(3-etilbenzotiazolina-6-ácido sulfônico), Sistema β-caroteno/ácido linoleico e atividade quelante dos íons Fe^{+2}, para respaldar seus possíveis mecanismos de ação;

- Avaliar o efeito mutagênico do extrato hidroalcoólico de *B. pilosa* nas concentrações de 100mg.kg^{-1}, 200mg.kg^{-1} e 300mg.kg^{-} por meio da análise da frequência de micronúcleos em células de medula óssea de camundongos albinos *Swiss* (*M. musculus*) *in vivo,* para as plantas no estágio de floração.

- Avaliar e comparar resultados obtidos por meio de análises de correlação.

3. ARTIGOS CIENTÍFICOS DERIVADOS DA TESE

3.1 MANUSCRITO 1

O manuscrito intitulado “Genetic and phytochemical variability of four Bidens pilosa L. populations and their bioactivity examined by antioxidant, mutagenic and antimutagenic approaches” foi submetido para avaliação ao periódico *Industrial crops and products*.

Genetic and phytochemical variability of four *Bidens pilosa* L. populations and their bioactivity examined by antioxidant, mutagenic and antimutagenic approaches

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Abstract

Bidens pilosa L. has been reported as a plant used in traditional medicine for treating a lot of disorders. The present study evaluated the variability of four population of *B. pilosa* using genetic marker (RAPD), phytochemical analysis (total phenols, total flavonoids and total tannins contents), antioxidant activity in vitro, cytotoxicity/anticytotoxicity and mutagenicity/antimutagenicity assays (by micronucleus test in bone marrow of mice) in order to determine factors that influence an accumulation of phenolic compounds and consequently their biological properties. Significant variability was detected between the hydroalcoholic extracts from different locations. Results strongly suggest that the variation of biological activities was caused by environmental rather than genetic factors.

Keywords: *Bidens pilosa*, RAPD, phytochemical analysis, antioxidant activity, micronucleus test, cytotoxicity.

1. Introduction

Different sources of dietary antioxidants may be especially important to delay, prevent or remove damage induced by oxidative stress, directly or indirectly (Landete, 2013; Halliwell, 2007a, 2007b). A good strategy to prevention and treatment of many degenerative diseases arising from mutations is the intake of substances whether natural or synthetic, such as antioxidants compounds, capable of preventing the formation or repair damage already constituted (Devasagayam et al., 2004; Rahman,2007; Tlili et al., 2014). Moreover, due to the possible adverse effect of synthetic antioxidants, which may have side-effects such as carcinogenic (Ebrahimabadi et al., 2010), food industries pay much attention to natural antioxidants, which can be used as good additives or as pharmaceutical supplements (Nicoli et al., 1999; Tlili et al., 2014).

Among the natural compounds, phytochemicals, such as phenolic compounds, are of great importance due by owning important biological activities, including anticarcinogenic, antimutagenic, antioxidant and antimicrobial (Valdés et al., 2015; Valdez-Morales et al., 2014; Attia, 2008). Consequently, progression of many chronic diseases, such as cardiovascular and neurological disorders, can be delayed by using phenolic compounds rich plants to some extent. However, the same substances presents in plant extracts could exhibit, both, antioxidant and pro-oxidant activity (Procházková et al., 2011; Dorman and Hiltunen, 2011) depend on the concentration, and biological system used. These properties make them also interesting as source of compounds where cell death, through apoptosis or necrosis induced via oxidative stress-related mechanisms is a desired outcome (Dorman and Hiltunen, 2011; Šamec et al., 2015).

Bidens pilosa is an herbaceous plant from South America widely distributed across temperate and tropical regions (Abdou et al., 2010). It is considered to be a rich source of food and medicine for humans and animals, and its cultivation were actively promoted in Africa by

Food and Agricultural Organization because it has the advantages of fast-growing, be edible, palatable, and safe (Bartolome et al., 2013).

This specie is used in traditional medicine for treatment of inflammation, allergy, diabetes, cancer, hepatitis and among others disorders (Arthur et al., 2012; Bartolome et al., 2013). Many biological and pharmacological analyses were tested. It has been reported its effectiveness immunomodulatory (Horiuchi and Seyama, 2008) anti-inflammatory (Alvarez et al., 1999; Yoshida et al., 2006; Pozharitskaya et., 2010), anti-hyperglycemic (Habeck, 2003), potential against malaria (Brandão et al., 1997; Oliveira et al., 2004; Kumari et al., 2009), as well as high cytotoxicity against various cell lines (Abdou et al., 2010; Tagami et al., 2009) including cancer cells (Kviecinski et al., 2008; Kumari et al., 2009; Sundararajan et al., 2006). Moreover, its phytochemical content and antioxidant activity has been widely studied (Chiang et al., 2004; Kumari et al., 2016; Ouerghemmi et al., 2016).

B. pilosa is an extraordinary source of phytochemicals, particularly flavonoids and polyynes (Silva et al., 2011; Bartolome et al., 2013). Despite having innumerous medicinal properties, researches linking their biological properties (such as antioxidant, cytotoxic, mutagenic and antimutagenic activities) and their contents of secondary metabolites with genetic variability and different locations of plant growth, not yet been carried out.

It is well known that the phytochemical compounds varies according several factors, such as environmental (the growth location of the plant, for example) and/or genetics (Chirinos et al., 2013; Tlili et al., 2014), which may affect their biological properties. The differences between the populations can be established by different markers; however chemical and molecular markers, used separately, are often insufficient to establish relations of variability among different populations (Morone-Fortunato et al., 2010; Trindade et al., 2009). RAPD, a dominant marker, has been successfully used to analyze the genetic diversity of natural populations (Ali et al., 2012; Singh et. al, 2012; Facanali et. al, 2015) due to its advantages

such as its potential for polymorphism detection without the need for prior knowledge of the genome, requires small amounts of genomic DNA and is able to detect changes in coding and noncoding regions (Gajera et al., 2010; Katsiotis et al., 2009; Lin et al., 2009).

There are no reports about influence of different geographical region and genetic variability on phytochemical content and antioxidant activity of B. pilosa. So, the goal of this work was to study the differences in amount of phenolic compounds, in antioxidant activity in vitro, in the cytotoxic/anticytotoxic and mutagenic/antimutagenic effects of B. bilosa hydroalcoholic extract, with respect to molecular markers and four geographical regions.

2. Material and methods

2.1 Plant material

The aerial parts of *Bidens pilosa* L. were randomly selected and collected at four localities of Southeastern Brazil region: Afonso Cláudio (AC) (41º 09′ 58.57″ W; 20º 15′ 07.33″S), Barra de São Francisco (BSF) (40º 54′ 51.9″ W; 18º 44′ 43.9″ S), Cariacica (CA) (40º 23' 54.0" W; 20º 17' 28.5" S) and Muniz Freire (MF) (41º 25′ 22.593″ W; 20º 31′ 38.1008″ S), during January 2014. The samples were air-dried at room temperature and then ground for further analysis. Vouchers specimens were identified, for all localizations, by the VIES herbarium of the Universidade Federal do Espírito Santo.

2.2 Genetic analysis using RAPD markers

For the genomic DNA extraction, 0.1 grams of 10 plants previously frozen of each locality were ground in liquid nitrogen in presence of polyvinylpolypyrrolidone. The obtained powder was mixed with 1 mL of CTAB extraction buffer (2% CTAB, 1.4M NaCl, 100 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0), β-mercaptoetanol and proteinase-K, incubated for 30 min at 65ºC, based on the procedure described by Doyle and Doyle (1990) with little modification. After cooling, 500 µL chloroform:isoamyl alcohol (24:1) are added to samples and

centrifuged for 5 min at 12000 RPM. This procedure is repeated twice. The supernatant was treated with RNAse A (10 µg.µl^{-1}), to 37ºC for 30 min. DNA precipitation was performed with 0.6% by volume of cold isopropanol. The formed pellet was washed with 70% ethanol, dried at room temperature and resuspended in 100 µL of Tris–EDTA pH 8.0 (TE Buffer). The DNA concentration was evaluated spectrophotometrically by NanoDrop 3300 (Termo Scientific), and the quality of the DNA was determined by electrophoresis on 1.0% agarose gel stained with GelRed™ (BIOTIUM™).

Initial PCR reactions were performed using thirty-two random decamer primers from the Operon Technology-USA, twelve- one primers (OPAD-01, OPAD-08, OPAD-10, OPAD-17, OPAD-18, OPD-04, OPD-18, OPG-19, OPI-14, OPI-19, OPI-20, OPE-06, OPE-11, OPE-12,OPP-05, OPP-06, OPP-08, OPP-09, OPP-10, OPF-18, OPF-20) have been selected on the basis of the good resolution and the polymorphism of bands. Each PCR reaction was performed in 25µL reaction volume containing 25 ng DNA template, 5 µl de 5x reaction buffer, 2.5 µL 25mM $MgCl_2$, 0.5 µl 10mM dNTP, 1.25U Taq and 0.3 µL (10 µM) of specific primer, in Veriti® 96-Well Thermal Cycler (Apllied Biosystems™). The PCR cycles following the conditions: 94◦C for 3 min, 40 cycles of desnaturation at 94◦C for 1 min, annealing at 35◦C for 1 min and extension at 72◦C for 2 min. After the cycles, the samples were submitted to a final extension step at 72◦C for 10 min. Amplification products were separated on 1% agarose gel in TBE buffer (1x), stained with GelRed™ (BIOTIUM™), visualized under UV light and recorded using a transilluminator LPIX-TOUCH (Loccus Biotecnologia, Brazil).

2.3 Preparation of hydroalcoholic extract

The plant powder was macerated with aqueous ethanol 70% using a solvent to powder ratio of ratio 5/1 (v/w) for 72h, at room temperature. The process was repeated twice with same powder to remove the maximum of constituents. Then, the resulting extracts were filtered

with a filter paper to remove the particles and concentrated under vacuum evaporator to obtain the crude hydroalcoholic extracts of *B. pilosa* (HAE).

2.4 Phytochemical screening

2.4.1 Phytochemical prospecting

The Phytochemical prospecting was carried out according to the method of Costa (1982) in order to identify secondary metabolites groups such as alkaloids, anthraquinone, coumarins, flavonoids, naphthoquinones, saponin, steroids, tannins and triterpenoids presents in crude HAEs from all localities.

2.4.2 Total phenolic content (TPC)

Total phenolic content (TPC) was measured carried out Zhang et al. (2006) by the Folin–Ciocalteu method. 20 µL ethanol solution of HAE 500 $\mu g.mL^{-1}$ was added to 100 µL of Folin–Ciocalteu diluted in distilled water (1:10). Then 5 minutes, 80 µL of Na_2CO_3 (7.5%) was added and the plate stayed in the dark at room temperature for 1 hour. The absorbance was measured at 750 nm with a spectrophotometric microplate reader (Epoch Microplate Spectrophotometer - BioTek). TPC was expressed as gallic acid equivalent per gram of dry weight (mg $GAE.g^{-1}$ d.w.). Concentrations of gallic acid used to establish the standard were 12.5, 25, 50, 10, 250, 500, 1000 $\mu g.mL^{-1}$ (R^2=0.9997). Ethanol was used as a blank. The analysis was run in in triplicate and conducted for the crude HAE extracts from all locations.

2.4.3 Total tannins content (TTC)

Total tannin content (TTC) of *B. pilosa* HAE was measured by the Folin–Denis method (Makkar et al., 1993; Ryu et al., 2016) with a few modifications. Previously, solution of 500 $\mu g.mL^{-1}$ of dry HAE was prepared in ethanol. 400 µL of ethanolic solution (500 $\mu g.mL^{-1}$) were mixed with 400 µL of Folin–Denis reagent. Then 3 minutes, 400 µL of Na2CO3 solution (8%) was added, mixed and allowed to stand. After an hour, the material was

centrifuged at 2000 rpm for 5 minutes and the absorbance measured at 725 nm. Tannic acid (concentrations 12.5, 25, 50, 100, 250, 500, 1000 µg.mL^{-1}) was used to calculate the standard curve (R^2= 0.9999) and the results were expressed as tannic acid equivalents (mg TA.g^{-1} d.w.).

2.4.4 Total flavonoid content (TFC)

Total flavonoid content (TFC) was quantified using the colorimetric method with aluminum chloride ($AlCl_3$) carried out according to Dewanto et al. (2002) and Tlili et al (2014), by spectrophotometric microplate reader. 250 µL of HAE diluted in methanol was mixed with 75 µL of $NaNO_2$ (7%) and after 150 µL of $AlCl_3$ (10%) was added and mixed. After 6 minutes, 500 µL of NaOH (1M) was added to the mixture and the absorbance was measured at 510 nm after 15min of incubation at room temperature. TFC was expressed as quercetin equivalent per gram of dry weight (mg QE.g^{-1} d.w.). Concentrations of quercetin used to establish the standard curve of flavonoids were 40, 50, 80, 100, 200, 300, 400, 500 µg.mL^{-1} (R^2=0.9828). The analysis was performed in triplicate and conducted for the extracts from all locations.

2.5 Antioxidant activity assays

The antioxidant activity of the HAE samples was measured in terms of radical scavenging activity, chelating activity and linoleic acid peroxidation inhibition, using four established spectrophotometric methods modified for microplate reader. All the experiments were performed in triplicate for each concentration tested.

2.5.1 Free radical-scavenging activity by DPPH assay

HAE and standards (ascorbic acid and hesperidin) was diluted in pure methanol at different concentrations (15.62, 31.25, 62.5, 125, 250, 500, 1000 µg.mL-1). 200 µL of methanolic DPPH solution (0.3 mM) was added to 100 µL of the test solution. After incubation for 30

min at room temperature in the dark, the decrease in absorbance was measured at 517 nm. A decrease in the absorbance indicates an increase in the scavenging activity.

Results were expressed as IC_{50} value ($\mu g.mL^{-1}$), which is the concentration of extract required to scavenges 50% of $DPPH^{\cdot}$. A lower IC50 value corresponds to a higher antioxidant activity. The percentage of inhibition of DPPH was calculated, with the following equation (Harzallah et al., 2016): **% inhibition of DPPH =** $[(Abs_o - Abs_1) / Abs_o] \times 100$, where Abs_o = absorbance of control and Abs_1 = absorbance of the sample.

Methyl alcohol it was used as blank, for the calibration spectrophotometric microplate reader. DPPH solution (0.3 mM; 200 µL) plus methyl alcohol (100 µL) was used as negative control.

2.5.2 Free radical-scavenging activity by $ABTS^{\cdot+}$ assay

The $ABTS^{\cdot+}$ assay was determined according Re et al. (1999) with small modifications. The work solution was prepared by mixing of 5 mL of 7 mM ABTS solution and 88uL of 140 mM potassium persulfate solution followed by incubation for 16 hours in the dark to yield a solution containing $ABTS^{\cdot}+$ radicals. Then, the solution was diluted with ethanol to absorbance value of 0.70 (±0.02) at 734 nm. 200 µL of work solution was added to 40 µL of the ethanolic solutions of crude HAE or the standards (Trolox and ascorbic acid) at eight concentrations (7.2, 15.62, 31.25, 62.5, 125, 250, 500, 1000 $\mu g.mL^{-1}$). The decrease in absorbance was measured at 734nm 6 minutes after mixing. The experiment was performed in triplicate for each concentration tested and the scavenging activity was estimated by the following formula: **% scavenging =** $[(Abs_o - Abs_1) / Abs_o] \times 100$, where Abs_o = absorbance of control and Abs_1 = absorbance of the sample. The results were expressed in mM equivalents of Trolox for gram of dry weight of HAE (TEAC; mM $TE.g^{-1}$ d.w.) and IC_{50} ($\mu g.mL^{-1}$).

2.5.3 Determination of chelating activity of HAE on Fe^{2+} ions

The ferrous ion-chelating activity of all HAE was measured using the method of Tang et al., (2002). 1 mL of HAE methanolic solution or EDTA standard (7.2 - 15.62, 31.25, 62.5, 125, 250, 500, 1000 µg.mL-1) was mixed with 22 µL of 2 mM FeCl2. Then, 43 µL of 5 mM ferrozine was added, homogenized and the absorbance was read at 562 nm in microplate reader after 20 min. The solution with Methanol (1 mL) instead of HAE or standard, was used as a control. The percentage of inhibition of ferrozine-Fe^{+2} complex formation was calculated as follows: **Chelating activity (%) =** (1 - Abs_1/Abs_o) x 100, where Abs_o = absorbance of control and Abs_1 = absorbance of the sample. The results were expressed in EDTA equivalent (mg EDTA.g^{-1} d.w.) and IC_{50} (µg.mL^{-1}).

2.5.4 Total antioxidant activity by the β-carotene/linoleic acid model system

For this assay, β-carotene was dissolved in $CHCl_3$ (0.5 mg.mL^{-1}) and to 1mL of this solution were added 80 µL of linoleic acid and 530 µL of Tween 40. The chloroform was fully evaporated for 30 minutes with oxygenator. 50 mL of oxygenated water was added, and the solution was shaken until all material dissolved. To each well of the microplate, 40 µL of crude HAE ethanolic solution or standards (Trolox, ascorbic acid, α-tocopherol or BHT) and 250 µL of the reagent mixture were added. Readings of all samples were taken at 470 nm immediately (t = 0 min) and after 120 min of incubation at 50ºC. The test mixture was prepared fresh and using immediately. The ethanolic solutions of HAE were tested at 250, 500 and 1000 µg.mL^{-1}. 40 µL of ethanol plus 250 µL of the reagent mixture was used as control. The antioxidant activity was calculated as percentage inhibition of oxidation in relation to the β-carotene control, using the following formula: % I= [(ΔAbs_o - ΔAbs_1) /ΔAbs_o] x100, where ΔAbs_o = absorbance initial - final of control and ΔAbs_1 = absorbance initial – final of the sample (Duarte-Almeida et al., 2006). The results were expressed as IC_{50} values (mg.mL^{-1}) and Trolox equivalent antioxidant capacity (TEAC mM TE.g^{-1} d.w.).

2.6 Mutagenic and antimutagenic activity

Mutagenicity and antimutagenicity was assessed by micronucleus assay in bone marrow of mice. The research was approved by the Research Ethical Committee on Animal Use of the Universidade Federal do Espírito Santo (CEUA/UFES, 026/2013) and was performed in accordance with the ethical principles of animal experimentation.

2.6.1 Animals and treatments

144 Swiss albino mice (*Mus musculus*), males, with 6–8 weeks of age and about 32 ± 4 g b.w., randomly selected and supplied by the biotery of the Universidade Federal do Espírito Santo were housed in plastic cages in groups of six animals, under conditions of controlled light and temperature, with free access to commercial feed and water.

2.6.2 Acute treatment: mutagenic and cytotoxic tests

The evaluation of crude HAE of *Bidens pilosa* as a cytotoxic and mutagenic agent was conducted for the all locations surveyed (AC, BSF, CA and MF). Thus, for each location we obtained five experimental groups: (i) the treated groups with a single dose of HAE dissolved in water, at final concentrations of 100, 200 and 300 $mg.kg^{-1}$ b.w., (ii) the positive control that received a single intraperitoneal injection (i.p) of cyclophosphamide (CPA; 50 $mg.kg^{-1}$, Sigma-Aldrich, St. Louis, Missouri, USA), and (iii) the negative control treated with a single dose of saline solution (0.9%). The treatments with HAE and saline were performed orally (gavage), once it is the form it's administered by the population. The animals were euthanized by displacement cervical 24 h after the treatment or application.

2.6.3 Subchronic treatment: Antimutagenic and anticytotoxic tests

The antimutagenic and anticytotoxic tests was carried out from the analysis of the phytochemical and antioxidant activity results, therefore, the following experimental groups were established: HAE from Cariacica (HAE CA) and Afonso Claudio (HAE AC).

The evaluation of HAE from CA and AC as a protect agent to genetic material was carried out using the pre-treatment protocol. For the two localities, the animals (n=6) were divided in the same five treatment groups (i, ii and iii) of the mutagenicity test. The pre-treated groups of Swiss albino mice received HAE by gavage (100, 200 and 300 $mg.kg^{-1}$, b.w.) once daily for 30 days and the clastogenic agent CPA (50 $mg.kg^{-1}$ b.w., i.p) 24h after the last dose of the extract (31^{th} day). The negative control was treated for 30 days with saline solution (0.9%), followed by administration of saline solution i.p on the 31^{th} day. The positive control received saline for 30 days, followed by administration of CPA (50 $mg.kg^{-1}$ b.w., i.p.), on the 31^{th} day. The animals were euthanized by displacement cervical 24h after the application.

2.6.4 Micronucleus assay in bone marrow cells

The micronucleus test in bone marrow of mice was performed as described by Schmid (1975) and MacGregor et al. (1987). After the smear drying, slides were stained with Leishman (Kinetics, 100% for three minutes and 1 Leishman:6 distilled water, for fifteen minutes), for the differentiation of blood cells, especially normochromatic erythrocytes (NCE), polychromatic erythrocytes (PCE), and micronucleated polychromatic erythrocytes (MNPCE), parameters of the analysis.

The mutagenic effect of HAE on DNA and its antimutagenic effect against cyclophosphamide-induced damage were determined by analyzing of 2,000 PCEs for each animal to determine the frequency of MNPCE. Its cytotoxic effect and its protective action against the cytotoxic effects of CPA were assessed by the ratio of PCE to 400 erythrocytes (PCE + NCE) per animal, using the formula PCE/(PCE + NCE). These analyses were performed according to the criteria established by Krishna and Hayashi (2000). The slides were analyzed in duplicate using optical microscope with increase of 1,000x (Nikon E200-LED, Nikon Instruments INC., New York City, New York, USA).

For the antimutagenicity test, the percent of damage reduction was calculated according to Waters et al. (1990) using the equation: **Damage Reduction (%)** = (MNPCE A - MNPCE B) / (MNPCE A - MNPCE C) x 100, where A is the group treated with saline solution more cyclophosphamide (positive control); B is the group treated with HAE solutions of *B. pilosa* more cyclophosphamide; and C is the group treated with saline solution (negative control).

2.7 Cytotoxicity *in vitro*

2.7.1 Lymphocytes isolation

Human lymphocytes were obtained from peripheral blood sample of a healthy nonsmoking volunteer with informed consent, aged between 20 and 30 years, without any history of recent disease, exposures to radiation or drug use (without alcohol ingestion thirty days prior blood donating). The lymphocytes were isolated by the traditional method in Ficoll® Paque Plus (Sigma–Aldrich) gradient, as recommended by manufacturer with minimal modifications. All protocols were approved by the Research Ethical Committee of UFES.

2.7.2 Cell culturing methods

Cells were cultured with RPMI 1640 (Cultilab) supplemented with antibiotic gentamicin (50 mg/L) and antimycotic amphotericin B (2 mg/L), 20% of fetal calf serum (Gibco) at 37 º and CO_2 5% saturation.

2.7.3 MTT assay

The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was performed to evaluate cell viability. The cells were plated in 96-well plates with 2x105 cells in each hole and treated with different concentrations of Bidens pilosa extract at dosage of 0 μg/mL, 12.5 μg/mL, 25 μg/mL, 50 μg/mL and 100 μg/mL, in triplicate, after 24 h of treatment, 20 μL MTT (Sigma–Aldrich) was added to each hole, and 3 h later, absorbance at 595 nm was detected in ELISA reader.

2.7 Statistical analysis

All results were expressed as the means ± standard error. Principal component analysis (PCA) and hierarchical cluster analysis (HCA) were carried out using XLSTAT (version 2016.05.33324) for Windows (Addinsoft, New York, USA), in order to obtain relationships between the four analyzed *B. pilosa* samples. For RAPD analysis, the presence of a band was scored 1, whereas the absence of the band was coded 0. The scored RAPD markers are converted into a binomial (0/1) matrix. From this data, the genetic proximity was estimated among the populations. Only polymorphic loci were used, applying Jaccard similarity coefficient. A dendrogram was built from the similarity analysis generated, using the Unweighted Pair-Group Method Arithmetic Average (UPGMA) method. For the micronucleus tests and cytotoxicity/anticytotoxicity effects, were performed ANOVA followed by Tukey test *a posteriori* at 5% of probability ($P < 0.05$); for antioxidant assays, were performed ANOVA followed test t ($P < 0.05$) using ASSISTAT version 7.7 beta software (Assistat Software, Campinas, São Paulo, Brazil).

3. Results

3.1. Genetic variability

The twelve selected primers generated 58 discernible and reproducible DNA fragments polymorphic. The amplified bands ranged between 100 and 2000bp. The UPGMA cluster analysis of the Jaccard's similarity coefficient generated a dendogram demonstrating the overall genetic relationship among the populations. Two groups were formed and there was low similarity between them (10,3%). In the groups, the lowest genetic similarity were found between populations MF and AC (2,2%) and higher among BSF and CA (58,1%), as shown in Fig. 1.

3.2. Phytochemical screening

The phytochemical composition, defined by the prospecting, TPC, TTC and TFC contents of HAE from AC, BSF, CA and MF, was estimated. The phytochemical prospecting showed positive results for flavonoids, cyaniding, coumarins, tannins and phytosterols (Liebermann-Burchard reaction) and showed the absence of alkaloids and triterpenes for all locations studied (Table 1). Naphthoquinones were identified only in HAE from CA and AC and saponins were identified in HAE from BSF and AC.

Due to their extensively reported antioxidant, pharmacological and toxicological data, the phenolic compounds shown in this study were evaluated by quantification of TPC, TTC and TFC, according presented in Table 2. There were significant differences ($P < 0.05$) between all HAE studied. Considering populations, highest levels of phenols, tannins and flavonoids were found in HAE CA (76.971 mg GAE.g^{-1}, 71.596 mg TAE.g-1 and 565.580 mg QE.g-1, respectively).

3.3. Antioxidant activity *in vitro*

The antioxidant capacity was evaluated using four complementary *in vitro* methods: DPPH and ABTS free radical scavenging activity, β-carotene-linoleic acid model and chelating activity on Fe^{+2}.

3.3.2 Free radical-scavenging activity

The scavenging ability of the HAE on DPPH and ABTS cation radicals is exhibited in Fig. 2 and Table 3. In these assays, the concentration-dependent profiles of scavenging power were observed for all extracts.

The results in DPPH assay was expressed as scavenging effect (%) and IC_{50} value (µg.mL^{-1}). The scavenging activity increased in the order of CA > BSF > MF > AC with effect of 86.78%, 70.49%, 60.22% and 51.65%, respectively, at the concentration of 500 µg.mL^{-1} (Fig.

2a). The standards ascorbic acid and hesperidin exhibited effects of 97.1% and 47.88%, in the same concentration of HAE reported. IC_{50} values of the extracts and controls are given in Table 3. The lower IC_{50} value indicates a stronger ability of the extract to act as a scavenger, while the higher IC_{50} value indicates that more extract is necessary to achieve 50% scavenging reaction (Tongpoothorn et al., 2012). Samples of CA showed a radical scavenging activity (IC_{50} 187.020 µg.mL^{-1}) significantly higher than the other locations (Table 3). This activity could be correlated to TFC (r^2=0.885) (Fig. 3a) and a moderate correlation was obtained with TPC (r^2=0.648), as shown in Fig 3b.

The antioxidant activity in ABTS assay was expressed as scavenging effect (%), IC_{50} value (µg.mL^{-1}) and Trolox equivalent antioxidant capacity (TEAC, mM TE.g^{-1} d.w.) (Tab.3). The scavenging activity increased in the order of CA > MF > AC > BSF with effect of 93.57%, 89.39%, 87.19% and 85.71%, respectively, at the concentration of 250µg.mL^{-1}. The standards ascorbic acid and Trolox exhibited effects of 94.48% and 94.09% (Fig.2b), at the same concentration. Samples of BSF showed a radical scavenging activity significantly higher (IC_{50} 54.944 µg.mL^{-1}), followed by CA (69,043 µg.mL^{-1}) (Tab. 3). The TEAC values demonstrated that CA showed higher amounts of Trolox per gram of extract (3.138 mM TE.g^{-1} d.w.), when compared with other locations (Tab.3). A correlation was obtained with TEAC value and TFC (r^2=0.864), as shown in Fig 3c.

3.3.3 Ferrous ion-chelating activity

The chelating activity on Fe^{2+} ions were expressed as scavenging effect (%), IC_{50} value (µg.mL^{-1}) and EDTA equivalent (mg EDTA.g^{-1} d.w.), as demonstrated in Table 3. In this trial, as in DPPH and ABTS assays, it was noted the increase of activity depending on concentration (Fig. 2c). The chelating activity increased in the order of AC > MF > CA > BSF with effect of 94.94%, 92.06%, 86.59% and 75.04%, respectively, at the concentration of 500µg.mL^{-1} (Fig. 2c). The standard EDTA exhibited 94.84% at the same concentration (Fig.

2c). HAE from AC showed significantly lower IC_{50} value (75.146 µg.mL^{-1}) and HAE from MF demonstrated higher EDTA equivalent value followed by AC, with 127.610 and 99.311 mg EDTA.g^{-1} d.w., respectively (Table 3). A moderate correlation was obtained with TTC (r^2=0.763) and TPC (r^2=0.658) (Fig. 3d, e). IC_{50} values of ABTS assay and chelating activity demonstrated correlation (r^2=0.883), as shown in Figure 3f.

3.3.4 β-carotene/linoleic acid model system

Heat-induced oxidation of a reagent mixture (model system of β-carotene and linoleic acid) was employed for this antioxidant test, to assess if the extracts inhibit lipid peroxidation. This method is based on the discoloration of the β-carotene due to peroxides generated during the oxidation of linoleic acid at elevated temperature, in the absence of antioxidant (Miller, 1971; Koleva et al., 2002 H. Hajlaoui, 2010). Thus, the degradation rate of β-carotene can be slowed down in the presence of antioxidants, depending on the activity of the extracts (Lu et al., 2014; Trabelsi, et al., 2013). The effects of *B. pilosa* on oxidation of β-carotene/linoleic acid at 50ºC was expressed as percentage of inhibition of lipid peroxidation (I %), IC_{50} (mg.mL^{-1}) and TEAC (mg TE.g^{-1} d.w.). HAE from MF exhibited better results than the HAE from the other locations for all parameter here analyzed, showing lower IC_{50} (1.841 mg.mL^{-1}) and higher Trolox equivalent value (0,434 mg TE.g^{-1} d.w.) (Tab. 3). A moderate correlation was obtained with TPC (r^2=0.619) and TTC (r^2=0.647) (Fig. 3 g, h). There were significant similarity observed in I (%) ($P < 0.05$) of HAE from MF and BSF, with percentage inhibition of 24.53% and 18.48%, respectively, at the concentration of 1000 µg.mL^{-1} (Tab. 4). Ascorbic acid exhibit a prooxidant activity (-39.28%) at the same concentration of the extract.

3.4 Correlations between phytochemical contents and different antioxidant assays: explorative analyses

An evaluation of the data was carried out with principal component analysis (PCA) to gain an overview of the similarities and differences among the populations and to investigate the

relationship among phytochemical contents and different antioxidant activity assays. PCA is a good instrument for viewing simultaneous analysis of many variables (TTC, TPC, TFC, DPPH, ABTS, chelating activity and β-carotene) and samples (populations).

The first and second principal components explained 70.4% and 20.52% of total variance (90.92%), respectively (Fig. 4). From the biplot, it was observed that HAE CA showed high and positive correlation with the amount of TFC, TPC, TTC and ABTS (mM TE.g-1 d.w.) and negative correlation with DPPH IC_{50} value ($\mu g.mL^{-1}$), highlighting that lower IC_{50} corresponds to a higher activity. These results indicate that TFC, TPC, TTC content and these two antioxidant assays were strongly correlated with each other. Flavonoids, phenols and tannins, more concentrated in HAE CA than in other locations studied, seem to be the principal contributors to strong the free radical-scavenging activity. PC2 mainly explained variation between samples with chelating activity (mg $EDTA.g^{-1}$ d.w.).

High correlation between TFC with ABTS and DPPH was further demonstrated by Pearson correlation analysis (DPPH: r^2=-0.941; ABTS: r^2=0.927). Chelating activity and inhibition of lipid peroxidation (β-carotene) were negatively correlated with TTC (Chelating: r^2=-0,874, β-carotene: r^2=-0,804) and TPC (Chelating: r^2=-0,811, β-carotene: r^2=0,787) (Table 7 - Supplementary information).

Figure 5 shows a hierarchical cluster analysis (dendrogram) based on antioxidant activity and phytochemical content of *B. pilosa* from different populations. This analysis showed that geo-location can significantly influence in variance of phytochemical contents and antioxidant activity. When comparing this grouping with grouping (Fig. 5) of the samples according to the RAPD analysis (Fig. 1), the results indicates that phytochemical and antioxidant variations among populations are caused by environmental factors rather than genetics.

2.5 Mutagenic, antimutagenic and cytotoxic activity

Table 5 summarize the results of the frequency of micronucleated polychromatic erythrocytes (MNPCE) and the ratio between the number of polychromatic (PCE) to normochromatic erythrocytes (NCE) in the bone marrow of mice treated with a single dose of HAE from all locations studied. All the groups (n = 6) treated with HAE did not increased the frequency of micronucleus compared to the positive group (Tukey, $p < 0.05$), indicating that HAE didn't have mutagenic effects under these experimental conditions. The analysis of the effects of HAE on the induction of cytotoxicity showed no significant alterations of the ratio PCE/(PCE + NCE) in all treated groups. Only HAE from Cariacica (300 $mg.Kg^{-1}$) exhibited significantly reduction of the ratio, indicating a possible cytotoxic effect in these experimental conditions (Tukey, $p < 0.05$).

The frequency of MNPCE, the ratio PCE/(PCE + NCE) and the percentage of damage reduction in the bone marrow of mice pre-treated with a daily single dose (for 30 days) of HAE from Afonso Claudio and Cariacica on cyclophosphamide-induced mutagenicity and cytotoxicity are shown in Table 6.

All groups treated with HAE AC significantly reduced the frequency of MNPCEs compared to the positive control group, indicating that this extract have antimutagenic activity under these experimental conditions (Table 6). The percentages of reduction of damages of the groups (n = 6) treated with HAE AC at concentrations of 100, 200 and 300 mg. kg^{-1}b.w were 27.72%; 36.83%; 53.29%, respectively, observed a dose-dependent response. The analysis of the effects of HAE AC on cyclophosphamide-induced cytotoxicity showed a significant increase of the ratio PCE/(PCE + NCE) in all treated groups (n = 6).

The groups treated with HAE from CA did not reduce significantly the frequency of micronucleus in all concentration studied (Tukey, $p < 0.05$). The analysis of the effects of HAE CA on cytotoxicity induced by cyclophosphamide showed a significant increase of the

ratio PCE/(PCE + NCE) only treated 300 mg. kg^{-1} group. No extract studied induced *in vitro* cytotoxicity, in these experimental conditions (Fig. 6).

4. Discussion

Dietary intake of phytochemicals may promote many benefits on human health, protecting against many degenerative disorders such as cancer, cardiovascular and neurodegenerative diseases (Sharma et al., 2014). Several studies have demonstrated the phytochemical content of *Bidens pilosa*, in especial, the flavonoids content has been widely investigated (Silva et al., 2011; Bartolome et al., 2013). Phenolic compounds, such as flavonoids, are considered the main actors for the antioxidant capacity of plants and have also many benefits on health attached to this property (Leopoldini et a., 2011; Tlili et al., 2014; Zhang and Tsao., 2016). Considering populations, the highest amount of phenolic compounds has been found in hydroalcoholic extract of plants grown at locality Cariacica and it was observed a high variation between the localities (Table 2).

Previous studies have shown that the phytochemicals content of plants, such as phenols, tannins and flavonoids, are influenced by numerous factors, such as genetic, geographical conditions, temperature, salinity, water, biotic or abiotic stress, sunlight and other conditions (Kähkonen et al., 1999; Lisiewska et al., 2006; Fratianni et al., 2007; Ksouri et al., 2008; Chirinos et al 2013; Baiano et al., 2013; Tlili et al., 2014). To assess the genetic variability between the populations, RAPD markers were evaluated. The RAPD provide a convenient and rapid tool in assessing genetic differences between genotypes, even at a lower intraspecific taxonomic level (Gad et al., 2013).

Levels of similarity between populations were between 58,1% and 33,3% (Fig. 1) showing that the populations are relatively different. *B. pilosa* is a species autogamous, display up to 10% of cross-fertilization and reproduces by seeds (Sun and Garders, 1990). It's distributed in all the cultivable regions of many countries and a single plant can produce 3.000 viable seeds

(Kissmann and Groth, 1999). These characteristics ensure high genetic variability of this species. The low level of similarity observed in the two clusters formed, indicate that clustering it is not only related with the proximity of geographic locations. Possibly, seed dispersal through migratory birds or another animals (zoochory) and with cultivated species seeds contaminated with *B. pilosa* facilitated gene flow by seed (Vidal et al. 2007), making possible that population from distant locations were grouped into a closer cluster due to their common origin. Vidal et al. (2006) also found high genetic variability among *Bidens* gender of plants from the same population. Lamego et al. (2006) and Vidal et al. (2007) founded low genetic similarity of *B. pilosa* from different populations.

B. pilosa HAE showed a great variability in TPC, TTC and TFC contents and consequently in antioxidant activity with respect to geographical region (Tab.3). Similar results were reported for other species (Mditshwa et al., 2013; Ouerghemmi et al., 2016; Ghasemzadeh and Jaafar, 2013; Kumari et al., 2016).

Antioxidants with free radical scavenging activities may have great relevance in the prevention and treatment of free-radical-mediated diseases (Hasan et al., 2009). DPPH and ABTS assays were used to investigate whether the extract could act by this mechanism. These methods have been used extensively to evaluate reducing substances and are useful reagents for investigating the free radical scavenging activities of compounds (Cotelle et al., 1996, Khoudja, 2014). In this study, it was observed that the extracts containing high levels of flavonoids were also potent radical scavenger (Fig 4 and Table 7), suggesting that may be the principal constituent responsible for the antiradical properties of the extract. This positive correlation could also be observed with *B. pilosa* (Wu et al., 2013; *Ibrahim et al., 2015)* and other plants (Clarke et al., 2013; *Cao et al., 2013;* Kumari et al., 2016).

Phenolic are chemical compounds that normally contribute to antioxidant potential of plants due to its unique structure characterized by at least one aromatic ring (C6) bearing one or

more hydroxyl groups, able to neutralize free radicals by forming resonance-stabilized phenoxyl radicals (Rice-Evans et al., 1996; Bors and Michel, 2002; Sakihama et al., 2002) when the phenolic antioxidant generate hydrogen atoms to stop the chain reaction of oxidation of lipids (Lindsay, 2010).

In addition, the four HAE studied showed better scavenging activity against ABTS radical than the DPPH radicals, showing lower IC_{50} values (Tab. 3). Indeed, the ABTS assay is more sensitive to identifying the antioxidant activity because of the faster reaction kinetics and a heightened response to antioxidants. Furthermore, the ABTS is soluble in aqueous and organic solvents, while DPPH is soluble only in organic (Lee et al., 2015).

Due to the wide variety of antioxidant components in the hydroalcoholic extract and the complexity of the oxidation-antioxidation processes, no single testing method is capable of providing a comprehensive picture of the antioxidant profile of a given sample (Khoudja et al 2014; Swapana et al., 2013). The analysis of chelating activity and inhibition of lipid peroxidation were carried out and negatively correlated with TTC and TPC (Tab. 7, supplementary material).

The results suggest that phenolic compounds present in the extracts are not the only factor affecting antioxidant activity. These data can be attributed to the structural factors of the individual antioxidants. The presence of other functional groups in the whole molecule, such as double bond conjugated to phenolic group and ketonic groups for example, which play different polarities in the structure of the antioxidant, can be attributed to their antioxidant activity (Erkan et al., 2008; Lu et al., 2014). Furthermore, the complex composition of antioxidants could provoke synergistic, additive or antagonistic actions between their components, although the mechanism of this effect is not understood completely (Lindsay, 2010; Koleva et al., 2002).

The difference of potential inhibition of lipid peroxidation (Tab. 4) it may be due to the greater presence of polar substances or due to structural features of the antioxidant. Antioxidants with less polarity exhibit stronger antioxidative properties in emulsions, as in β-carotene/linoleic acid assay, because they have higher affinity with the lipidic side of the system, thus ensuring high protection of the emulsion itself. On the other hand, polar antioxidants remaining in the aqueous phase are less effective in lipids (Koleva et al., 2002). The ascorbic acid showed prooxidant activity in β-carotene/linoleic acid system, corroborating previous research (Jayasinghe et al., 2013; Putchala et al., 2013; Carocho and Ferreira, 2013). This effect can be observed in HAE from CA and AC at lower concentration. The phenolic compounds in the reduced form act as an antioxidant. In contrast, in the oxidized form, phenoxyl radical (OH•), produced through antioxidative reactions can exhibit prooxidant activities (Sakihama et al., 2002). The OH• has a high reactivity due to its very short half-life, hardly may be kidnapped *in vivo* (Lone et al., 2013). There are two ways to control the OH• radical presence: repair the damage caused by it or inhibit their formation (Barreiros and David, 2006).

Free radicals, such as OH•, are known to cause damage to many biomolecules and cell structures. The high concentration of Oxigen-reactive species (ROS) can damage cellular lipids, proteins or DNA, inhibiting their normal function (Valko, 2007). Because of this, oxidative stress has been implicated in a number of human diseases as well as in the ageing process. The permanent modification of DNA resulting of ROS represents the first step involved in mutagenesis, carcinogenesis, and ageing (Sharma et al., 2012; Valko et al., 2006).

The extracts from all locations showed no mutagenic and no cytotoxic activity at the concentrations tested, indicating that can’t induce DNA damage or cell death (Tab. 5). Cyclophosphamide, a chemotherapeutic agent that exerts its therapeutic function via alkylation, was used to induce genomic damage and cytotoxicity (positive control). It can

generate visible damage to DNA due to oxidative stress resulting from free radical production and decay of enzymatic antioxidant levels (El-Bayoumy, 2001; Manda and Bhatia, 2003; Sugumar et al., 2007). Furthermore, CPA induces lipid peroxidation (Manda and Bhatia, 2003; Azevedo et al., 2010) and cause greater increase in serum iron (Fe II e III). These ions are potentially harmful as it is the main catalyst of the reactions of free radicals *in vivo* (Garófolo (2011).

Several factors are associated with increased oxidative stress in patients with cancer and that make the use of chemotherapy. Because of this, oxidative stress has been implicated in a number of human diseases as well as in the ageing process. The delicate balance between beneficial and harmful effects of free radicals is a very important aspect of living organisms and is achieved by mechanisms called “redox regulation” (Valko, 2007).

Like this, the possible antimutagenic and anticytotoxic potential of HAE was examined against CPA for two locations, HAE from CA, with the highest amount of phenolic compounds and higher scavenging activity, and HAE from AC, with the lowest amount of flavonoids and higher chelating activity (Tab. 2 and 3). These locations are considerably different between them (Fig 4 and 5).

No antimutagenic effects were found in groups treated with HAE CA, while HAE AC exhibits antimutagenic effects. The strongest antimutagenic activity was observed at 300 mg.Kg-1. This result can be due to a greater ability to chelate iron ions that HAE AC showed. One of main mechanisms of HO• formation is by reaction of hydrogen peroxide with transition metals in a low oxidation state, like Fe (Fenton and Haber-Weiss reactions) (Sakihama, 2002; Barreiros and David, 2006; Leopoldini et al., 2011).

The removal of free transition metals in biological environment is fundamental for antioxidant protection of the organism. Thus, it can be concluded that HAE from Afonso Claudio acts by desmutagen mechanism, preventing the constitution of damage (mutation) and cancer

initiation process by blocking reactive species either by scavenging, electron donation or through chelation and thus maintains the DNA structure (Devi et al., 2015; Leopoldini et al., 2011). The protective agents can act directly on the compounds which induce DNA damage, inactivating them chemically or enzymatically, before they act on the genes Thus, in general, all the antioxidant agents are potential inhibitors of mutagenesis and carcinogenesis (Oliveira et al., 2009; Ferguson, 1994; Bhattacharya, 2011).

Xenobiotic phenolics are known by modulate the antioxidant response of the cell, through enzymatic stimulation (Shetty and Wahlqvist, 2004; Calou, 2009). But, these compounds can exert genotoxic and mutagenic effects as a result of their prooxidant properties (Flowers et al., 1997). The groups treated with HAE CA in the mutagenic assay demonstrated the possible cytotoxic effect, in the high concentration. Furthermore, the groups treated with HAE AC exhibits protective effects against the cytotoxicity induced by CPA. Probably, the effectiveness of the protection against cytotoxicity by these antioxidants depends primarily on their rate of incorporation into cells due to their lipophilicity, secondly on their antioxidant activity, and thirdly on their orientation in biomembranes (Kaneko, 2001). This protective effect could not be observed in the groups treated with HAE from Cariacica (100 and 200 $mg.Kg^{-1}$).

HAE CA and HAE AF were the only ones with naphthoquinones. Cariacica exhibited the most amount of this natural pigment (strong reaction). These compounds have important biological activities. Among them, have antioxidant capacity and ability to inhibit DNA topoisomerase; this type of mechanism may be involved in the cytotoxic activity of naphtoquinones (López et al., 2014).

Similar to our research, many studies performed on different cell lines, including tumor cells, suggest that *B. pilosa* extract induced apoptosis cytotoxic action (Chang et al., 2001; Chiang et al., 2004; *Sundararajan et al.,2006;* Kviecinski et al., 2008, 2011; Wu et al., 2013;

Chavasco et al., 2014; Kumari et al., 2009). Costa et al. (2008) demonstrated the presence of mutagenic effects *in vitro*, when administered in high concentration. Furthermore, the cytotoxicity activity was correlated positively with the total flavonoid contents, as shown Wu et al (2013). Given that HAE CA exhibited higher content of flavonoids, it's possible that their cytotoxic effect is due to this correlation. This reinforces the need for caution, especially when using high doses for a long period. the cytotoxicity activity was correlated positively with the total flavonoid contents, as shown Wu et al. (2013). Given that HAE CA exhibited higher content of flavonoids, it's possible that their cytotoxic effect is due to this correlation. This reinforces the need for caution, especially when using high doses for a long period.

This research represent the first comparative molecular, phytochemical and antioxidant analyzes on relationship between *B. pilosa* populations by chemometric approach, composed of hierarchical cluster analysis (HCA) and principal component analysis (PCA). Our results demonstrated differences in the clusters when were analyzed the genetic marker (RAPD) and antioxidants/phytochemicals data. This suggests that genetic factors do not interfere significantly on the phytochemical production and antioxidant activity. Similar results were observed with other species, demonstrating that factors related to geographic location outweigh the genetic factors (Wang et al., 2009; Granato et al., 2010; Harzallah, et al., 2016; Chirinos et al., 2013; Padula et al., 2013; Schlag and McIntosh., 2013).

5. Conclusion

This comparative study based on preliminary phytochemical, antioxidant and molecular analyses of four *B. pilosa* populations from Brazil showed that environmental are crucial in determining phenolic contents rather than genetic variability. The results of this study indicate that the biological properties of the extract, under the same preparation conditions, can change according to environmental growing conditions. Future studies to standardize the cultivation and production of the extract should be performed.

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Conflicts of interest

The authors declare no conflict of interest.

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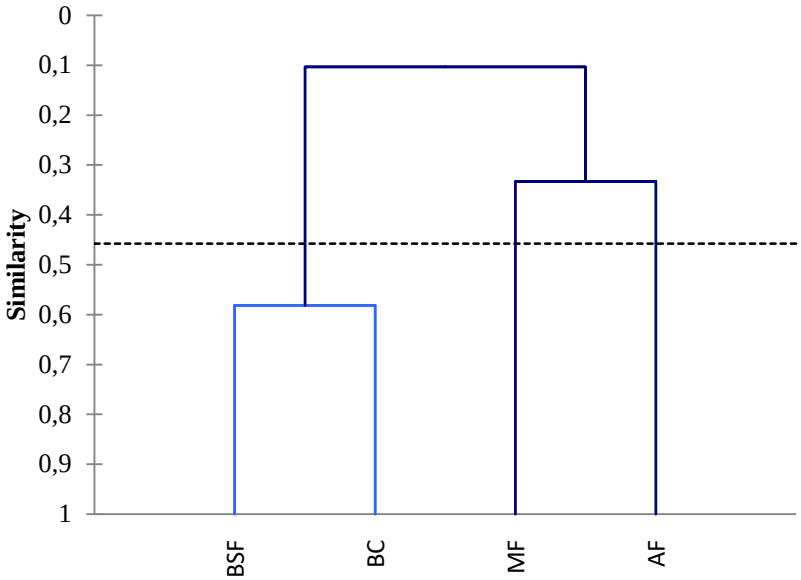

Fig. 1. Dendrogram showing genetic variability between four *Bidens pilosa* populations determined by RAPD markers. BSF, Barra de São Francisco; CA, Cariacica; MF, Muniz Freire; AC, Afonso Claudio.

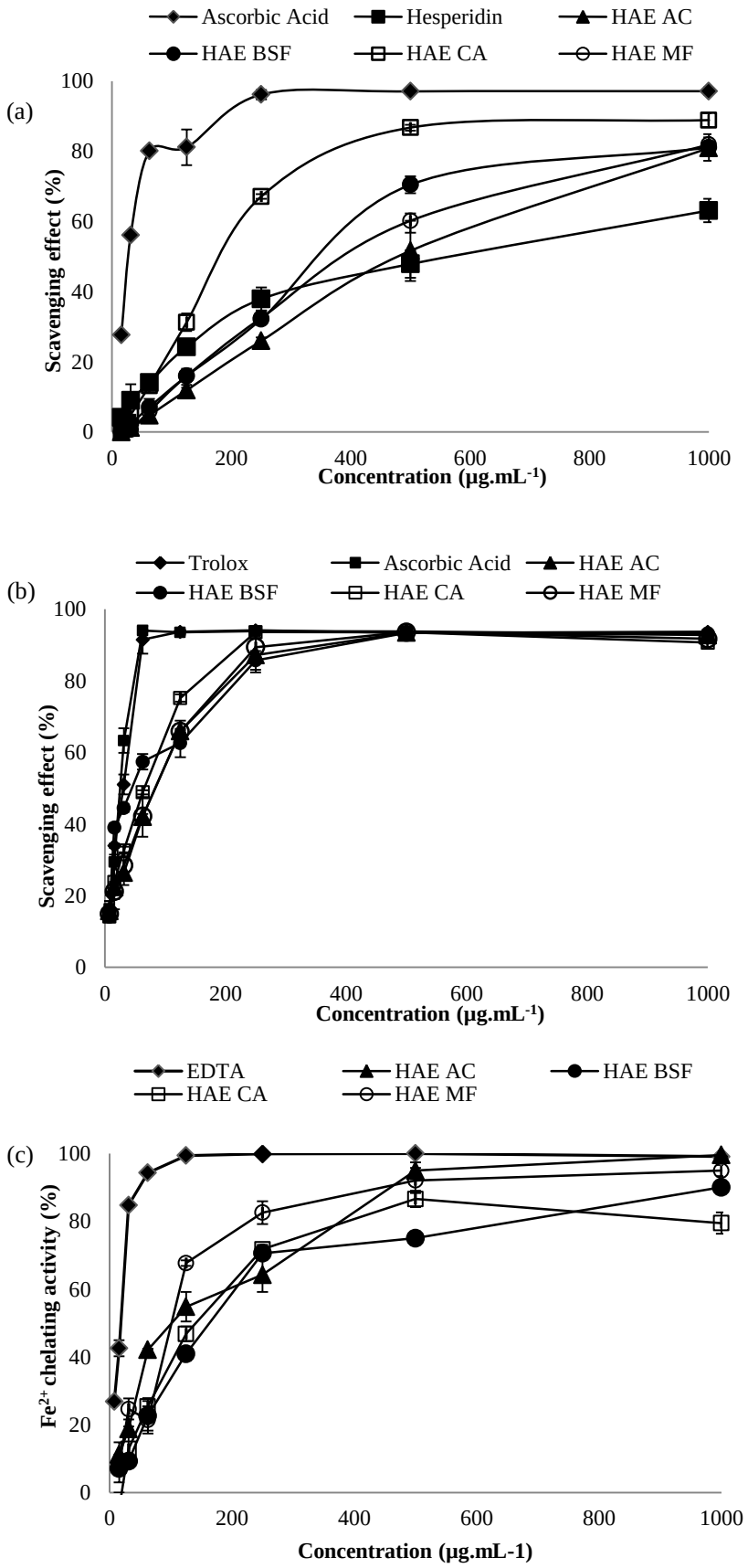

Fig. 2. Antioxidant activity of hydroalcoholic extract of *Bidens pilosa* from four locations and standards (ascorbic acid, hesperidin, Trolox and EDTA) shown by percentage of scavenging effect and ferrous chelanting activity. (a) DPPH radical scavenging activity; (b) ABTS radical scavenging activity (c) Fe^{2+} chelating activity. AC, Afonso Claudio; BSF, Barra de São Francisco; CA, Cariacica; MF, Muniz Freire. All the values are expressed as mean ± SE (n=3); SE: standard error.

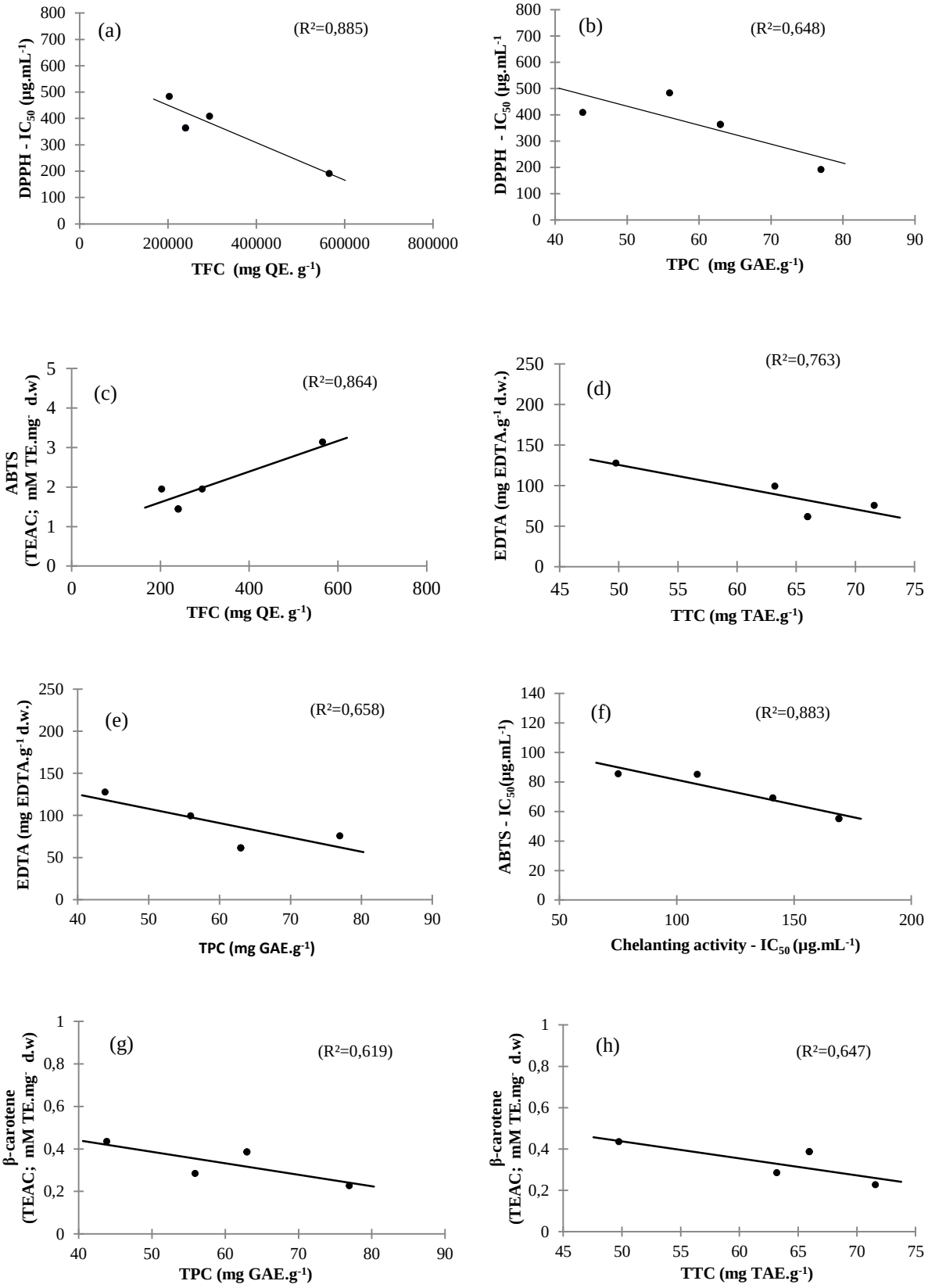

Fig. 3 Relationship between the contents of constituents in the extracts and scavenging activity by DPPH (a,b), ABTS (c), Ferrous ion-chelating activity (d, e), and Trolox equivalent (β-carotene/linoleic acid assay). The Relationship between ABTS and Chelating activity (IC_{50}) was demonstrated in (f). TFC, total flavonoids content; TPC, total tannins content; QE, quercetin equivalent; GAE, acid gallic equivalent; TEAC, Trolox equivalent antioxidant capacity.

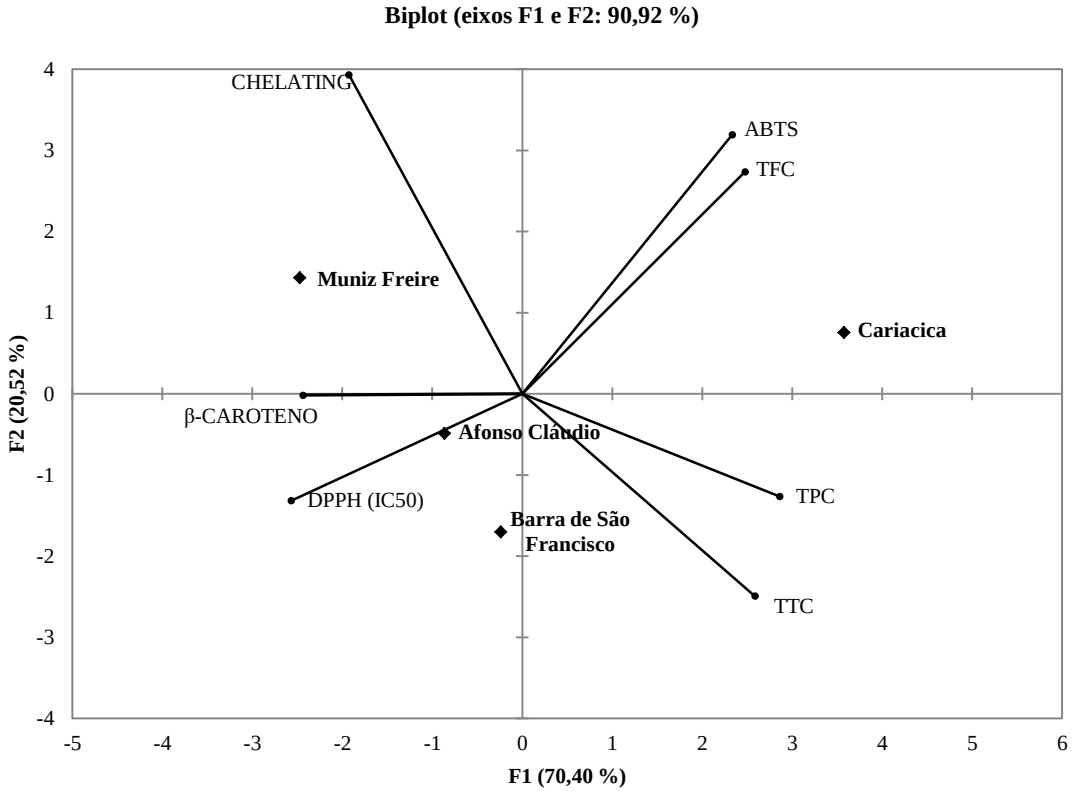

Fig. 4. Principal component analysis (scores and loading plots, biplot) based on different phytochemical compounds analyzed in hydroalcoholic extracts of *B. pilosa* from four populations and ther antioxidant activity (DPPH, Chelating activity – EDTA equivalent, Trolox equivalente - ABTS and β-carotene). TFC, total flavonoids content; TPC, total phenols content; TTC, total tanins content.

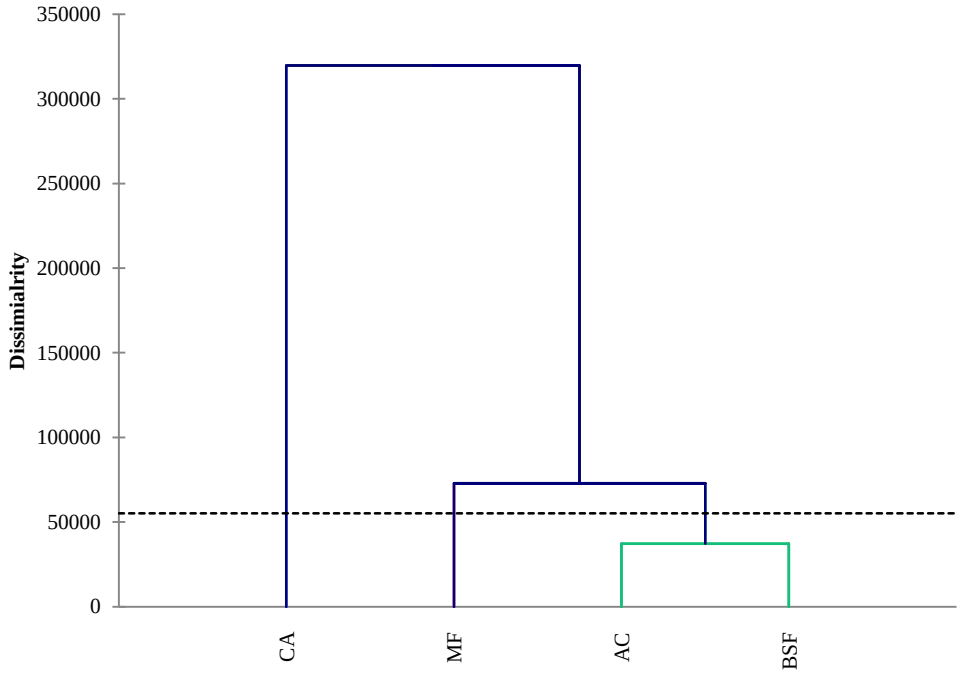

Fig. 5 Hierarchical cluster analysis based on phytochemical content and antioxidant activity of HAE from four B. pilosa populations that grew at different locations.CA, Cariacica; MF, Muniz Freire; AC, Afonso Claudio; BSF, Barra de São Francisco.

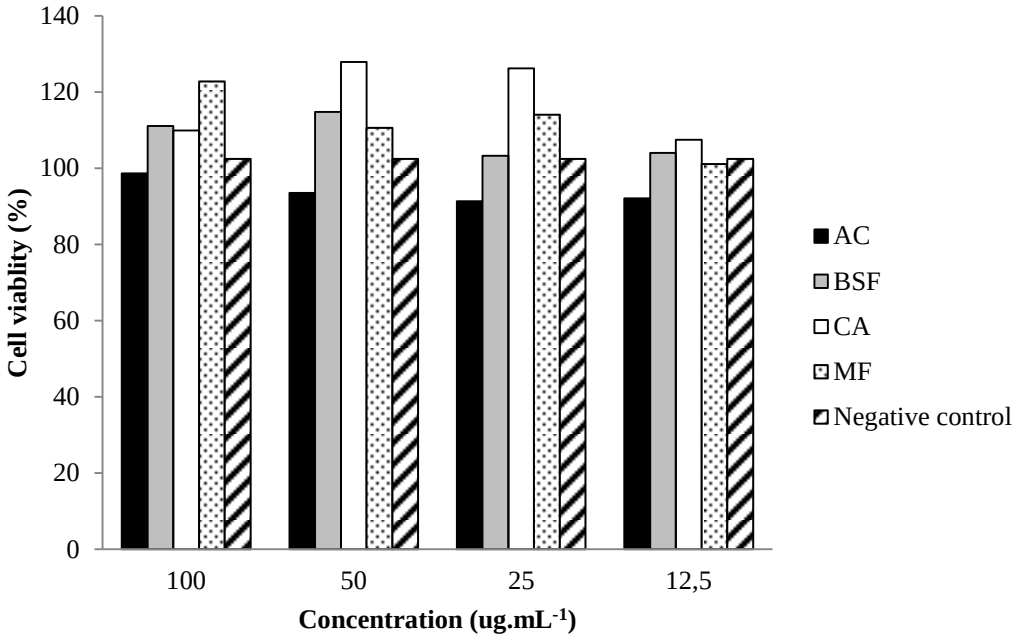

Fig. 6 Results obtained by the methyl tetrazolium (MTT) assay in lymphocytes human cells after exposure to hydroalcoholic extracts of *B. pilosa* from four populations at dosage of 0, 12.5, 25, 50 and 100 μg.mL^{-1}, for 24h; data presented as percentage (n=3).

Table 1. Phytochemical components of *Bidens pilosa* based on the hydroalcoholic extract from four locations: Afonso Claudio, Barra de São Francisco, Cariacica and Muniz Freire.

Phytochemical constituents	AC	BSF	CA	MF
Flavonoids	+	+	+	+
Cyanidin	+	+	+	+
Coumarins	+	+	+	+
Steroids	+	+	+	+
Triterpenes	-	-	-	-
Alkaloids	-	-	-	-
Tannins	+	+	+	+
Naphthoquinone	+	-	+++	-
Saponins	-	+	+	-

+: presence of reaction; +++: strong reaction; −: Nondetected; AC, Afonso Claudio; BSF, Barra de São Francisco; CA, Cariacica; MF, Muniz Freire.

Table 2. Content of phenolic compounds of *Bidens pilosa* hydroalcoholic extracts collected from four locations: Afonso Claudio (AC), Barra de São Francisco (BSF), Cariacica (CA) and Muniz Freire (MF).

Plant extract	Location	TPC (mg GAE.g^{-1} ± SE)	TTC (mg TAE.g^{-1} ± SE)	TFC (mg QE. g^{-1} ± SE)
HAE	AC	55.941^{c} ± 2.401	63.206^{b} ± 1.324	203.233^{d} ± 8.089
	BSF	63.000^{b} ± 2.401	65.978^{b} ± 1.792	240.329^{c} ± 5.775
	CA	76.971^{a} ± 1.321	71.596^{a} ± 0.619	565.580^{a} ± 1.623
	MF	43.882^{d} ± 2.857	49.760^{c} ± 0.520	294.648^{b} ± 5.299

All the values are expressed as mean ± SE (n=3); SE: standard error; TTC: Total tannins content; TPC: total phenols content; TFC: total flavonoids content. $^{a\text{-}d}$Means with same superscripts type indicated no significant difference, ANOVA, test-t ($p < 0.05$).

Table 3. Antioxidant activities of *Bidens pilosa* hydroalcoholic extract from four populations.

Plant extract/ chemical	Location	Antioxidant activity							
		DPPH IC_{50} ($\mu g.mL^{-1}$)	ABTS		Chelating activity		β-carotene linoleic acid	
			TEAC (mM Trolox.g^{-1} D.E)	IC_{50} ($\mu g.mL^{-1}$)	EDTA Equivalent (mg EDTA.g^{-1} D.E)	IC_{50} ($\mu g.mL^{-1}$)	TEAC (mM Trolox.g^{-1} D.E)	IC_{50} ($mg.mL^{-1}$)
Ascorbic acid	-	$35,170^{f}$ ± 0,88	2,117	$24,686^{d}$ ± 0,35	-	-	n.d*	n.d*
Hesperidin	-	$633,67^{a}$ ± 1,42	-	-	-	-	-	-
Trolox	-	-	-	$29,313^{d}$ ± 0,73	-	-	-	182,28
BHT	-	-	-	-	-	-	1,129	375,57
α-tocoferol	-	-	-	-	-	-	1,719	164,56
EDTA	-	-	-	-	-	$17,67^{e}$ ± 0.25	-	
HAE	Afonso Cláudio	$482,629^{b}$ ± 3,67	1,947	$85,333^{a}$ ± 2.51	99,311	$75,146^{d}$ ± 1.44	0,283	2.952^{a} ± 0.203
	BSF	$362,600^{d}$ ± 4,36	1,443	$54,944^{c}$ ± 5.18	61,428	$169,157^{a}$ ± 2.21	0,385	2.103^{b} ± 0.104
	Cariacica	$187,020^{e}$ ± 6,40	3,138	$69,043^{b}$ ± 2.02	75,487	$140,989^{b}$ ± 5.55	0,226	3.636^{a} ± 0.217
	Muniz Freire	$407,734^{c}$ ± 10,8	1,942	$77,621^{a}$ ± 0.65	127,610	$108,803^{c}$ ± 2.74	0,434	1.841^{b} ± 0.049

All the values are expressed as mean ± SE (n=3); SE: standard error; $^{a-e}$Means with same superscripts type indicated no significant difference, ANOVA, test-t ($p < 0.05$). n.d, not detected value; HAE, hidroalcoholic extract; *Prooxidant activity.

Table 4. Antioxidant activity (%) of the hydroalcoholic extracts of *Bidens pilosa* from four locations by β-carotene/linoleic acid assay, expressed as percentage of inhibition peroxidation.

Hydroalcoholic extracts/standards	**% of inhibition peroxidation**[a]		
	Concentration (μg.mL^{-1})		
	250	500	1000
α-tocoferol	59.535	62.126	70,963
Ascorbic acid	-15.998	-31.132	-39.288
BHT	54,286	59.668	66.711
Trolox	58.054	65.297	71.280
Afonso Cláudio	-12.525	-4.983	5.249
BSF	-3.115	7.346	18.486
Cariacica	-9.364	-3.862	3.950
Muniz Freire	1.708	11.354	24.532

[a]All the values are expressed as mean ± SE (n=3);

Table 5. Frequency of micronucleated polychromatic erytrocytes (MNPCE) in 1000 PCE and ratio between the number of polycromatic and normocromatic erythrocytes, by the formula PCE/(PCE + NCE), following the protocol of mutagenicity with hidroalcoholic extract of *Bidens pilosa* collected from four locations.

Treatment	Locations							
	Afonso Claudio		Barra de São Francisco		Cariacica		Muniz Freire	
	MNPCE/1000 PCE ± SE	Ratio (PCE:PCE + NCE) ± SE	MNPCE/1000 PCE ± SE	Ratio (PCE:PCE + NCE) ± SE	MNPCE/1000 PCE ± SE	Ratio (PCE:PCE + NCE) ± SE	MNPCE/1000 PCE ± SE	Ratio (PCE:PCE + NCE) ± SE
NaCl 0.9%[1]	0.916^{b} ± 0.271	0.545^{b} ± 0.007	0.916^{b} ± 0.271	0.545^{b} ± 0.007	0.916^{b} ± 0.271	0.545^{a} ± 0.007	0.916^{b} ± 0.271	0.545^{b} ± 0.007
Cyclophosphamide[2]	56.08^{a} ± 2.672	0.449^{a} ± 0.012	56.08^{a} ± 2.672	0.449^{a} ± 0.012	56.08^{a} ± 2.672	0.449^{b} ± 0.012	56.08^{a} ± 2.672	0.449^{c} ± 0.012
HAE 100mg.kg^{-1} b.w	0.083^{b} ± 0.083	0.536^{b} ± 0.013	0.083^{b} ± 0.083	0.578^{b} ± 0.008	0.083^{b} ± 0.083	0.512^{a} ± 0.006	1.583^{b} ± 0.490	0.602^{ab} ± 0.016
HAE 200mg.kg^{-1} b.w	4.083^{b} ± 0.790	0.514^{b} ± 0.012	3.250^{b} ± 0,972	0.555^{b} ± 0.011	0.916^{b} ± 0.300	0.502^{a} ± 0.009	2.000^{b} ± 0.790	0.549^{b} ± 0.011
HAE 300mg.kg^{-1} b.w	1.916^{b} ± 0.539	0.521^{b} ± 0.007	1.666^{b} ± 0,421	0.561^{b} ± 0.008	0.083^{b} ± 0.083	0.49^{ab} ± 0.021	1.500^{b} ± 0.342	0.609^{a} ± 0.019

Equal letters represent statistical similarity (ANOVA, Tukey test, $P < 0.05$). SE = standard error. [1]negative control; [2]positive control

Table 6. Frequency of micronucleated polychromatic erythrocytes (MNPCE) in 1000 PCE, ratio between the number of polychromatic and normochromatic erythrocytes, by the formula PCE/(PCE + NCE) and the percentage of reduction of the damages induced by cyclophosphamide, following the protocol of pre-treatment with hydroalcoholic extract of *Bidens pilosa* collected from two locations.

Treatment	Locations					
	Afonso Cláudio			Cariacica		
	MNPCE/1000 PCE ± SE	Ratio (PCE:PCE + NCE) ± SE	Reduction (%)	MNPCE/1000 PCE ± SE	Ratio (PCE:PCE + NCE) ± SE	Reduction (%)
NaCl 0.9%[1]	2.750^{d} ± 0.423	0.566^{a} ± 0.011	-	2.666^{b} ± 0.715	0.535^{ab} ± 0.013	-
Cyclophosphamide[2]	68.833^{a} ± 2.211	0.444^{c} ± 0.012	-	61.416^{a} ± 2.791	0.469^{c} ± 0.006	-
HAE 100mg.kg^{-1} b.w	50.333^{b} ± 3.544	0.516^{ab} ± 0.015	27,722	49.083^{a} ± 5.734	0.519^{abc} ± 0.012	21,023
HAE 200mg.kg^{-1} b.w	44.333^{bc} ± 4.187	0.508^{b} ± 0.009	36,835	62.250^{a} ± 3.057	0.499^{bc} ± 0.020	n.d.
HAE 300mg.kg^{-1} b.w	33.500^{c} ± 2.655	0.508^{b} ± 0.015	53,291	53.833^{a} ± 4.043	0.557^{a} ± 0.011	12,92613636

Equal letters represent statistical similarity (ANOVA, Tukey test, $p < 0.05$). SE = standard error. [1]negative control; [2]positive control; n.d., not detected value.

Table 7. Pearson Correlation analyses between phenol contents and antioxidant activities.

TTC, total tannins content; TPC, total phenols content; TFC, total flavonoids content; ChA, Chelating activity;

Variables	TTC	TPC	TFC	ChA (IC_{50})	ChA ($EDTA.g^{-1}$)	DPPH (IC_{50})	ABTS (IC_{50})	ABTS (TEAC)	β-CAROTENO (TEAC)	β-CAROTENO (IC_{50})
TTC	**1**									
TPC	0,956	**1**								
TFC	0,481	0,707	**1**							
ChA (IC_{50})	0,417	0,515	0,334	**1**						
ChA ($EDTA.g^{-1}$)	-0,874	-0,811	-0,235	-0,725	**1**					
DPPH (IC_{50})	-0,597	-0,805	-0,941	-0,628	0,493	**1**				
ABTS (IC_{50})	-0,600	-0,603	-0,180	-0,940	0,897	0,500	**1**			
ABTS (TEAC)	0,452	0,630	0,927	-0,024	-0,060	-0,763	0,125	**1**		
β-CAROTENO (TEAC)	-0,804	-0,787	-0,583	0,124	0,414	0,484	0,020	-0,751	**1**	
β- CAROTENO (IC_{50})	0,772	0,791	0,672	-0,105	-0,368	-0,558	0,002	0,827	-0,992	**1**

3.2 Manuscrito 2

O manuscrito intitulado “Influence of phenological stages and fertilizers on growth, chemical composition and biological activities of *Bidens pilosa* L.” será submetido para avaliação ao periódico *Journal of Pharmacy and Pharmacology*.

Influence of phenological stages and fertilizers on growth, chemical composition and biological activities of *Bidens pilosa* L.

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Abstract

The aerial part of *Bidens pilosa*, an herbaceous weed popularly known as "picão-preto", has medicinal properties such as for treatment of inflammation and hepatitis. The study was conducted to compare growth, secondary metabolites and biological activities (antioxidant, cytotoxic and mutagenic activities) of this specie in response to different phenological stages (vegetative and reproductive) and two sources of fertilizers: organic, with bovine manure (1:1, v:v), and inorganic fertilizer, with NPK (4-14-8 kg.ha−1). It was observed that the fertilizers enhanced the growth of the plants. But, the production of total tannins, phenols and flavonoids increased in the absence of fertilizer (control) with consequent higher antioxidant activity. The antioxidant activity was evaluated by three mechanisms of action: free radical scavenging (by DPPH and ABTS assays), lipid peroxidation inhibitory (by β-carotene/linoleic acid system) and ferrous ion-chelating activities. The results indicated that the use of fertilizer, under these experimental conditions, not induced cytotoxicity and mutagenicity *in vivo*, by micronucleus test in bone marrow of mice.

Keywords: *Bidens pilosa*; chemical fertilizer; organic fertilizer; antioxidant activity; micronucleus assay

1 Introduction

Plants have long been used for medicinal purposes for the treatment of human diseases [1,2]. It is observed the increasing attention to medicinal plants as source of chemicals, especially secondary metabolites with therapeutic and others actions of interest for the food and pharmaceutical industry [3, 4].

Research has shown the role of some secondary metabolites as protective constituents against some diseases. There is evidence that the daily intake of low doses of secondary metabolites, such as flavonoids, may reduce the incidence of cancers and many chronic diseases, including cardiovascular disease and type II diabetes [3]. However, the biosynthesis of phytochemical compounds are strongly influenced for numerous factors [5,6,7, 8, 9, 10, 11, 12, 13]. Among them, plant nutrition is one of the most important factors that affect quantitatively and qualitatively the secondary metabolites in plants and its growth. Other important factor is the phenological stage of harvesting [14] since the quantity and/or quality of active constituents is not constant during the year [8]. Like this, it is important to know how such conditions can affect the production of the metabolites, in order to maximize the yield of active constituents with greater benefits [9, 15, 16, 17, 18].

Bidens pilosa L. (Asteraceae), popularly known as "picão-preto", is a herbaceous weed originating in South America and widely distributed in tropical and subtropical regions of the world, mainly in agricultural areas (19, 20]. The plant was reported to possess several medicinal properties like for treatment of inflammation, jaundice, hepatitis, diabetes and cancer [20, 21, 22]. The aerial part of this plant was reported to possess antioxidant properties [23, 24, 25, 26, 27] cytotoxic activity against some cell lines [28, 29, 30, 31, 19) and others pharmacological effects attributed to its phytochemical composition, especially phenolic compounds (flavonoids) [32, 22]

Although it possesses innumerable medicinal properties, studies that relate its phenological stages and growth conditions with their phytochemical constituents and biological properties, such as antioxidant, cytotoxic and mutagenic activities, have not been performed to date. Hence, the present study was conducted to examine the effects of harvesting stage (vegetative and reproductive stages) and organic and chemical fertilizers source (bovine manure and NPK) on the growth, chemical composition and biological activities of *B. pilosa*. The relationships between these parameters were also investigated.

2 Methods

2.1 Reagents

The chemical reagent DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid), β-carotene, potassium persulfate ($K_2S_2O_8$), ferrozine (3-(2-Pyridyl)-5,6-di(2-furyl)-1,2,4-triazine-5′,5″-disulfonic acid disodium salt), Folin-Ciocalteu's phenol reagent, Folin-Denis, α-tocopherol, trolox and cyclophosphamide (CPA) was purchased from Sigma-Aldrich, USA. The others reagents were purchased from xxxxxxxxxx. CPA was used as the positive control substance due to its potential as DNA damaging agent in the micronucleus test *in vivo*.

2.2 Plant material and growth analysis

Seeds of *Bidens pilosa* L. were obtained from plants collected in Afonso Claudio, Brazil (41º 09′ 58.57″ W; 20º 15′ 07.33″ S). The field experiment was conducted in beds during 2014 at Muniz Freire, Brazil (41º 25′ 26.24″ W; 20º 31′ 34.87″ S) to study the influence of phenological stages and organic and inorganic fertilizers on growth, chemical composition, polyphenols content, antioxidant activity, cytotoxicity and mutagenicity of *Bidens pilosa* L.

The material was originated from plantations private locals, who does not require specific permissions and does not involve endangered or protected species.

Three conditions of experimental soil were tested: (i) the control with absence of fertilization was a red sandy loam having pH 6.3; (ii) the organic fertilizer, using bovine manure (1:1, v:v); and (iii) the chemical fertilizer , using mineral fertilizer (N), phosphorus (P) and potassium (K) 4-14-8 $kg.ha^{-1}$, respectively. All treatments were collected in two harvesting stages: vegetative stage and reproductive stage (flowering stage). Before plant culture, the soil was analyzed by the Agronomic Analysis Laboratory and Consulting LTDA - FULLIN (Linhares/ES, Brazil), methodology as EMBRAPA [33] (Supplementary Information).

The organic and chemical fertilizers were applied as per treatment before planting in the plots of 6.0 m^2 and incorporated into control soil. The seeds were sown directly on the plots for germination on 09th August, 2014. Crops were irrigated once a day with 24 liters of irrigation water, during all experiment. The plants were harvested in the vegetative stage on October, 2014 and the reproductive stage on november, 2014. For the growth analyses, the experiment was laid out in a randomized block design. The plots were divided in seven blocks with six repetitions. Every plant in each treatment was weighed and some measures were taken: height, stem diameter and leaf area before being placed in paper bag and dried at room temperature for 10 days to determine dry weight. It was used Image J free software to analyze the leaf area from digital images of the fresh plants. All growth analyses were performed in both phenological stages.

2.3 Hydroalcoholic extract

For the other analyzes, dry plants from vegetative and reproductive stages were used in the production of the hydroalcoholic extracts (HAE). The plant powder underwent exhaustive

maceration to remove the maximum of constituents, with aqueous ethanol 70% using a solvent to powder ratio of ratio 5/1 (v/w) for 72h, at room temperature. To obtain the crude HAE of B. pilosa, the resulting solution was filtered with a filter paper to remove the particles and concentrated under vacuum evaporator, resulting in six HAE.

2.4 Phytochemical prospecting

Preliminary staining and precipitation tests were performed according to [34] to identify secondary metabolites groups such as alkaloids, flavonoids, steroids and tannins presents in the HAE of plants from growth conditions and phenological stages.

2.4 Determination of total phenolic content (TPC)

Total phenolic content (TPC) was determined according to the method described by [35] with minor modification. Briefly, 20µL ethanol solution of HAE 500µg.mL^{-1} or standard solution of gallic acid at 12.5, 25, 50, 10, 250, 500, 1000µg.mL^{-1} (R^2=0.9997) was added into a test tube containing 100µL of Folin-Ciocalteu reagent diluted in distilled water (1:10). The mixtures were stirred and allowed to stand for 5 minutes. Then, 80µL of Na_2CO_3 (7.5%, w/v) was added and the plate stayed in the dark at room temperature for 60 minutes. The absorbance was measured at 750nm using UV-VIS spectrophotometric microplate reader (Epoch Microplate Spectrophotometer - BioTek). The TPC in each extract were determined as mg of gallic acid equivalent per gram of dry weight (mg GAE.g^{-1} DW) by using the regression equation from the calibration curve of the gallic acid standard. Ethanol was used as a blank. All determinations were performed in triplicate.

2.5 Determination total tannins content (TTC)

To determine the total tannin content (TTC) the Folin–Denis method was used [36, 37] with a few modifications. 400µL of ethanolic solution (500µg.mL^{-1}) of dry HAE of each treatment were mixed with 400µL of Folin–Denis reagent. The solution was vortexed and allowed to 3 minutes. Then, 400µL of Na_2CO_3 solution (8%, w/v) was added, mixed and allowed to stand for 60 minutes. After, the material was centrifuged at 2000 rpm for 5 minutes and the absorbance measured at 725nm using UV-VIS spectrophotometric microplate reader. The TTC expressed as mg tannic acid equivalent per gram of dry weight (mg TAE/g DW), was determined using a tannic acid curve at 12.5, 25, 50, 100, 250, 500, 1000 µg.mL^{-1} (standard curve, R^2= 0.9999).

2.6 Determination total flavonoids content (TFC)

Total flavonoid content (TFC) was estimated using the method reported by [38] and [13]. 250µL of methanolic solution (500 µg.mL^{-1}) was mixed with 75µL of $NaNO_2$ (7%, w/v) and then 150µL of $AlCl_3$ (10%, w/v) was added and mixed. After 6 minutes, 500µL of NaOH (1 M) was added to the solution. The mixture was allowed to stand for 15 minutes at room temperature and the absorbance was measured at 510nm using UV-VIS spectrophotometric microplate reader. TFC was expressed as mg of quercetin equivalent per gram of dry weight (mg QE.g^{-1} DW) calculated with respect to quercetin standard curve at 40, 50, 80, 100, 200, 300, 400, 500µg.mL^{-1} (R^2=0.9828). The analyses were performed in triplicate for all treatments.

2.7 Free radical-scavenging activity by DPPH and ABTS assays

For the DPPH assay, 100 µL of ethanolic solution of the HAE and standard (ascorbic acid) at 15.62, 31.25, 62.5, 124, 250, 500, 1000 µg.mL^{-1} were added to 0.3 mM DPPH in methanol,

and the reaction mixtures were shaken vigorously. The amount of remaining DPPH radical was determined at 517nm in UV-vis spectrophotometric micro plate reader after incubation for 30 min at room temperature in the dark, and the radical-scavenging effect was calculated as follows [39]: % **inhibition** of DPPH = $[(Abs_o - Abs_1) / Abs_o] \times 100$, where Abs_o and Abs_1 are the respective absorbances of samples without (control) and with extracts or standards solution. The ABTS radical scavenging measurements were performed according to the method of [40] with small modifications. The radical cation was prepared by mixing of 5mL of 7mM ABTS stock solution with 88uL of 140mM potassium persulfate solution followed by by incubation for 16 hours in the dark to yield a solution containing $ABTS^{\cdot}+$ radicals. The ABTS•+ solution was diluted in ethanol to an absorbance of 0.70 ± 0.02 at 734 nm, resulting the work solution. Then, 200µL of work solution was added to 40µL of the ethanolic solutions of the extracts or standard (Trolox) at eight concentrations (7.2, 15.62, 31.25, 62.5, 124, 250, 500, 1000µg.mL^{-1}). The antioxidant activity was calculated by determining the decrease in absorbance 6 minutes after mixing using the same DPPH equation. Results were expressed as IC_{50} value (µg.mL^{-1}), which is the antiradical dose of extract required to scavenges 50% of $DPPH^{\cdot}$. A lower IC50 value corresponds to a higher antioxidant activity. Methyl alcohol and ethanol it was used as blank, for the calibration spectrophotometric microplate reader. The experiment was performed in triplicate for each concentration tested.

2.7.2 Chelating activity on ferrous (Fe^{+2}) ions

The chelating effect on ferrous ions of the prepared extracts and the standard EDTA was estimated by the method of [41] with slight modifications. 1 mL of methanolic solution of each test sample or standard (EDTA), at 7.2, 15.62, 31.25, 62.5, 124, 250, 500, 1000µg.mL^{-1}, was mixed with 22µL of 2mM $FeCl_2$. The reaction was initiated by the addition of 43 µL of 5 mM ferrozine into the mixture which was then allowed for 20 min at room temperature and

then the absorbance was determined at 562 nm in UV-VIS spectrophotometric microplate reader. The percentage of inhibition of ferrozine-Fe^{+2} complex formation was calculated as follows: **Chelating activity (%) =** (1 - Abs_1/Abs_o) x 100, where Abs_o is the absorbance of control and Abs_1 is the absorbance of the samples. The results were expressed in IC_{50} value ($\mu g.mL^{-1}$).

2.7.3 β-carotene/linoleic acid model system

0.5 mg of β-carotene dissolved in 1 mL of chloroform ($CHCl_3$) (0.5$mg.mL^{-1}$ solution), 80µL of linoleic acid and 530µL of Tween 40 (polyoxyethylene sorbitan monopalmitate) were mixed together. The chloroform was completely evaporated for 30 minutes with oxygenator and the resulting solution was dissolved with 50mL of oxygenated water. 250µL of this reagent mixture were transferred into each well of the microplate containing 40µL of samples at 125, 250, 500 and 1000$\mu g.mL^{-1}$ or standards (Trolox, BHT and ascorbic acid) at 7.2, 15.62, 31.25, 62.5, 124, 250, 500, 1000µg.mL-1 concentrations in ethanol. Readings of all samples were taken at 470nm immediately (t = 0 min) and after 120 min of incubation at 50ºC. The antioxidant activity (AA) was calculated in terms of percent inhibition relative to the control using the formula: I(%) = [(ΔAbs_o - ΔAbs_1) /ΔAbs_o] x100, where ΔAbs_o is the absorbance initial – final of control and ΔAbs_1 is the absorbance initial –final of the sample [42]. The results were expressed as percent inhibition (I%) and Trolox equivalent (mM $TE.g^{-1}$ dw).

2.8 Mutagenic and cytotoxic activity *in vivo*

Mutagenicity and cytotoxicity was assessed by micronucleus assay in bone marrow of mice for hydroalcoholic extracts obtained from the plants harvested at the reproductive stage, for all tested fertilizer conditions, since it is the stage most used for medicinal purposes. The

experiments *in vivo* were performed in accordance with ethical principles of animal experimentation approved by the Research Ethical Committee on Animal Use of the Universidade Federal do Espírito Santo (CEUA/UFES, 026/2013).

2.8.1 Animals and treatments

66 Swiss albino mice (*Mus musculus*), male, was supplied and randomly selected by the biotery of the Universidade Federal do Espírito Santo with 6–8 weeks of age and about 30±4 g b.w. They were housed in plastic cages under conditions of controlled light and temperature, with free access to water and food. The evaluation of HAE as mutagenic and cytotoxic agent to genetic material was carried out using an acute treatment, with a single dose. Thus, for each fertilizer condition (control, organic and chemical fertilizer) five experimental groups were obtained: the treated groups with a single dose of hydroalcoholic extracts (HAE) dissolved in water at final concentrations of 100, 200 and 300 $mg.kg^{-1}$ b.w, orally by gavage; the negative control group, treated with a single dose of saline (0.9%, gavage); and the positive control group, that received a single intraperitoneal injection (i.p) of cyclophosphamide (CPA; 100 $mg.kg^{-1}$).

2.8.2 Micronucleus assay in bone marrow cells

The animals were euthanized by displacement cervical 24 h after the treatment and the slides of bone marrow cells were prepared according to [43]. After the smear drying, the slides were fixed in methanol P.A for 10 minutes and stained with Leishman's eosine methylene blue, for the differentiation of blood cells, especially polychromatic erythrocytes (PCE), micronucleated polychromatic erythrocytes (MNPCE) and normochromatic erythrocytes (NCE). The slides were analyzed using optical microscopy (Nikon E200-LED, Nikon

Instruments INC., New York City, New York, USA) with increase of 1000×. A total of 2000 PCE were analyzed per animal to determine the MNPCE frequency and the mutagenic effect. For assess the cytotoxic effect, was evaluated the ratio of PCE/(PCE + NCE) obtained from the analysis of 400 erythrocytes (PCE+NCE). All the analysis followed the criteria established by [44].

2.9 Statistical analysis

For growth analysis, results were expressed as the means ± standard desviation and the statistical analyses were performed by ANOVA for blocks with replications, followed by t-test ($p < 0.05$). The statistical analyses of micronucleus test and cytotoxicity effect were performed by ANOVA followed by Tukey test ($p < 0.05$), using ASSISTAT version 7.7 beta software (Assistat Software, Campinas, São Paulo, Brazil, http://www.assistat.com/). For antioxidants assays, the statistical analysis was performed separating the phenological stages (vegetative and reproductive) and to compare the responses to treatments between the phenological stages of the same fertilizer, by t-test ($p < 0.05$), using ASSISTAT. Correlation analyses were performed using software XLSTAT (version 2016.05.33324).

3 Results and discussion

3.1 Effects of fertilizer on grown parameters

Data presented in Table 1 reveal that plants submitted to organic and inorganic fertilizers increased significantly the leaf area, stature, fresh weight and dry weight. Plant fresh weight of organic and inorganic fertilizers increased by 187.66% and 151.36% over control (no fertilizer) in vegetative stage, and 135.02% and 110.16%, in the reproductive stage. No

significant difference was found between the plants fresh weight in organic and inorganic fertilizers (test-t, $p < 0.05$) for this parameter analyzed.

The organic and inorganic fertilizers increased the dry weight by 40.68% and 42.37% over control of vegetative stage, and 35.2% and 46.39% over control of reproductive stage. Furthermore, plant stature increased by 51.47% and 36.95% for organic and inorganic fertilizers in reproductive stage. Statistical difference between bovine and chemical fertilizers was found only for the leaf area of the reproductive stage and the stature of vegetative stage, where the organic fertilizer exhibited better results (t-test, $p < 0.05$).

Table 1. Effects of phenological stages and organic and inorganic fertilizers on growth parameters of *Bidens pilosa* L.

Phenological stages	Treatments (fertilizers)	Fresh weight (mg ± SD)	Dry weight (mg ± SD)	Leaf area (cm^2 ± SD)	Stature (cm ± SD)
Vegetative	Control	5.51 ± 0.56[b]	1.77 ± 0.08[b]	3.86 ± 0.87[b]	7.27 ± 0.59[c]
	Organic fertilizer	15.85 ± 3.19[a]	2.49 ± 0.23[a]	10.45 ± 2.96[a]	18.29 ± 1.58[a]
	Chemical fertilizer	13.85 ± 2.30[a]	2.52 ± 0.18[a]	9.69 ± 2.26[a]	15.09 ± 1.84[b]
Reproductive	Control	44.86 ± 6.82[b]	15.43 ± 1.51[b]	6.39 ± 2.19[c]	43.95 ± 4.44[b]
	Organic fertilizer	105.43 ± 24.16[a]	20.86 ± 2.27[a]	19.38 ± 3.48[a]	66.57 ± 16.72[a]
	Chemical fertilizer	94.28 ± 23.4[a]	22.58 ± 4.28[a]	11.46 ± 4.67[b]	60.19 ± 6.49[a]

All the values are expressed as mean ± SD (7 blocks with 6 replications); SD: standard deviation; [a-c]Means with same superscripts type indicated no significant difference into the same phenological stage, ANOVA, test-t ($p < 0.05$).

3.2 Phenological and fertilizer changes in major chemical parameters

Table 2 summarizes the results of phenological stages and fertilizers influences in changes of the total tannins (TTC), total phenols (TPC) and total flavonoids (TFC) contents. The TTC, TPC and TFC of *B. pilosa* hydroalcoholic extracts (HAE) significantly varied according to the different fertilizers used for growth and phenological stages (vegetative and reproductive). The highest TTC, TPC and TFC were found in the HAE of the control, in both phenological stages. In the vegetative stage, HAE obtained from *B. pilosa* of bovine and chemical

fertilizers didn't differ statistically. TTC and TPC of chemical fertilizer increased in the reproductive stage, while TFC increased for HAE of control and chemical fertilizer.

The phytochemical prospection showed positive results for flavonoids, coumarins, phytosterols (Liebermann-Burchard reaction) and naphtoquinones and showed the absence of triterpenes and alkaloids for all HAE studied (Table 3).

Table 2. Content of phenolic compounds of *Bidens pilosa* hydroalcoholic extracts obtained from plants of different phenological stages (vegetative and reproductive stages) and different fertilizers.

Phenological stage	Fertilizer	TTC (mg TAE/g SE)	TPC (mg GAE/g SE)	TFC (mg QE/g SE)
Vegetative stage	Control	67.63 ± 2.51^{a}	125.76 ± 1.30^{a}	359.68 ± 7.53^{a*}
	Organic fertilizer	34.63 ± 2.76^{b}	91.33 ± 6.08^{b}	237.34 ± 4.98^{b}
	Chemical fertilizer	42.27 ± 3.26^{b*}	89.02 ± 2.35^{b*}	240.48 ± 3.32^{b*}
Reproductive stage	Control	65.38 ± 0.66^{a}	133.31 ± 4.98^{a}	454.42 ± 1.66^{a*}
	Organic fertilizer	39.84 ± 1.93^{b}	98.82 ± 3.54^{b}	252.40 ± 3.07^{c}
	Chemical fertilizer	56.43 ± 1.63^{c*}	108.41 ± 4.12^{b*}	276.87 ± 4.98^{b*}

All the values are expressed as mean ± SE (n=3); SE: standard error; TTC: Total tannins content; TPC: total phenols content; TFC: total flavonoids content. $^{a-c}$Means with same superscripts type indicated no significant difference into the same phenological stage, ANOVA, test-t ($p < 0.05$). *Statistically significant difference between phenological stages of the same fertilizer (test-t, $p < 0.05$).

Table 3 Phytochemical components of *Bidens pilosa* based on the hydroalcoholic extract two phenological stages (vegetative and reproductive) and three growing conditions (control, chemical and organic fertilizers).

Phytochemical constituents	Vegetative stage			Reproductive stage		
	C	CF	OF	C	CF	OF
Flavonoids	+	+	+	+	+	+
Coumarins	+	+	+	+	+	+
Steroids	+	+	+	+	+	+
Triterpenes	-	-	-	-	-	-
Alkaloids	-	-	-	-	-	-
Naphthoquinone	+	+	+	+	+	+

+: presence of reaction; −: Non detected; C: Control, CF: Chemical fertilizer, OF: Organic fertilizer.

3.3 Antiradical activity

The *B. pilosa* HAE from all experimental conditions showed concentration dependent free radical scavenging activities as assayed by $DPPH^{\bullet}$ and $ABTS^{+\bullet}$ (data not shown). The free radical scavenging activities of all samples were reported as IC_{50} values (Table 4).

The $DPPH^{\bullet}$ scavenging activities showed highest value in the HAE of the control, in both phenological stages, with IC_{50} = 299.04 and 329.32 for vegetative and reproductive stages, respectively. The $ABTS^{+\bullet}$ antioxidant capacity showed highest activity for HAE from control, in both phenological stages, with IC_{50} = 71.32 and 79.27 for vegetative and reproductive stages, respectively. Samples of HAE from chemical and organic fertilizers showed radical scavenging activity significantly higher at the reproductive stage when compared with the vegetative stage for both scavenging tests (t-test, $p < 0.05$).

Increasing total tannins, phenols and flavonoids contents were accompanied by stronger antioxidant capacities of *B. pilosa* HAE of all treatments from vegetative stage (Table 4). DPPH scavenging activity was significantly correlated with TTC (r^2 = 0.999), TPC (r^2 = 0.907) and TFC (r^2 = 0.9474), already the ABTS scavenging activity was significantly correlated with TTC (r^2 = 0.8519), TPC (r^2 = 0.9866) and TFC (r^2 = 0.9626). In the reproductive stage, DPPH positive correlations were observed for TTC (r^2 = 0.9165) and ABTS for TTC (r^2 = 0.9995), TPC (r^2 = 0.8155) and TFC (r^2 = 0.747) (Table 5).

3.4 Ferrous ion-chelating activity

The chelating activity on Fe^{+2} ions from all experimental conditions showed concentration dependent activities (data not shown) and the results were expressed as IC_{50} value ($\mu g.mL^{-1}$), as demonstrated in Table 4. HAE from chemical fertilizer showed significantly lower IC_{50} value in both phenological stages, with IC_{50} = 207.13 and 132.86 for vegetative and reproductive stages, respectively. The chelating activity was better in reproductive stage for all treatments (control, chemical and organic fertilizer), with lower IC_{50} values when compared with vegetative stage (t-test, $p < 0.05$). A significant correlation was obtained with TTC (r^2 = 0.9853) and a moderate correlation with TPC (r^2 = 0.7324) and TFC (r^2 = 0.6103), for reproductive stage, as shown Table 5.

3.5 Inhibition of linoleic acid peroxidation

The capacity of HAE assess the inhibition of lipid peroxidation was dose dependent for all experimental conditions (Table 6). The results were expressed as percentage of inhibition of lipid peroxidation (I%) (Table 6) and TEAC value (µg Trolox.mL^{-1}) (Table 4). The extract of the control obtained in the vegetative stage presents a better TEAC (0.314) followed by the extract of the chemical fertilizer obtained in the reproductive stage (0.291). A significant correlation was obtained with TTC for vegetative (r^2 = 0.834) and reproductive (r^2 = 0.7627) stages. A moderate correlation with TFC (r^2 = 0.6623) for vegetative stage was found (Table 5).

3.6 Cytotoxicity and Mutagenicity *in vivo*

The PCE/(PCE+NCE) ratio and the micronucleated polychromatic erythrocytes (MNPCE) frequency are shown in Table 7. All groups (n = 6) treated with HAE from different conditions of growth (absence of fertilizer and presence of bovine or chemical fertilizer) collected in reproductive stage showed no significant cytotoxic and mutagenic activities compared to the positive control group (Tukey, $p < 0.05$), in these experimental conditions.

Table 5 Correlations between the IC_{50} values of antioxidant activities by DPPH, ABTS and Chelating assays, TEAC value (mM Trolox.g^{-}1) by β-carotene/linoleic acid assay, tannins, phenolics and flavonoids contents of *B. pilosa* hydroalcoholic extracts obtained from plants of different phenological stages (vegetative and reproductive stages) and different fertilizers (control, chemical and organic).

Phenological stage	**Assays (IC_{50} µg.mL^{-1} or mM Trolox.g^{-1})**	**Correlations r^2**		
		TTC	**TPC**	**TFC**
Vegetative stage	DPPH	0.999	0.907	0.9474
	ABTS	0.8519	0.9866	0.9626
	Chelating activity	0.0698	0.0001	0.0045
	β-carotene/linoleic acid	0.834	0.5861	0.6623
Reproductive stage	DPPH	0.9165	0.5701	0.44
	ABTS	0.9995	0.8155	0.747
	Chelating activity	0.9853	0.7324	0.6103
	β-carotene/linoleic acid	0.7627	0.3568	0.238

TTC: Total tannins content; TPC: total phenols content; TFC: total flavonoids content.

Table 4. Antioxidant activities of *Bidens pilosa* hydroalcoholic extracts obtained from plants of different phenological stages (vegetative and reproductive stages) and different fertilizers (control, chemical and organic).

Phenological stage/ chemical	Treatment (fertilizer)	DPPH	ABTS	Chelating activity	β-carotene linoleic acid
		IC_{50} (µg.mL^{-1})	IC_{50}, (µg.mL^{-1}±SE)	IC_{50} (µg.mL^{-1}±SE)	TEAC (Mm TE.g^{-1}±SE)
Ascorbic acid	-	38.54^{a} ± 0.41	±	-	n.d*
Trolox	-	-	24,27^{a} ± 0.60	-	-
BHT	-	-	-	-	1.129
EDTA	-	-	-	17.67^{a} ± 0.25	-
Vegetative stage	Control	299.04^{b} ± 15.55	71.32^{b} ± 1.92	241.00^{b*} ± 1.55	0.314
	Chemical	864.78^{c*} ± 14.30	272.10^{c*} ± 1.31	270.03^{c*} ± 2.87	0.270
	Organic	1064.09^{d*} ± 21.88	235.49^{d*} ± 8.57	207.13^{d} ± 3.13	0.203
Reproductive stage	Control	329.32^{b} ± 0.64	79,27^{b} ± 0.53	206.64^{b*} ± 1.32	0.277
	Chemical	364.43^{b*} ± 21.45	151,60^{c*} ± 9.14	189.01^{c*} ± 1.43	0.291
	Organic	857.69^{c*} ± 11.37	298,01^{d*} ± 4.13	132.86^{d*} ± 6.54	0.207

All the values are expressed as mean ± SE (n=3); SE: standard error; $^{a\text{-}e}$Means with same superscripts type indicated no significant difference into the same phenological stage, ANOVA, test-t ($p < 0.05$); *Statistically significant difference between phenological stages of the same fertilizer, ANOVA, test-t ($p < 0.05$); n.d, not detected value; *Prooxidant activity.

Table 6 Percentage of inhibition of lipid peroxidation (I%) of *Bidens pilosa* hydroalcoholic extract obtained from plants of different phenological stages (vegetative and reproductive stages) and different fertilizers, by β-carotene/linoleic acid system.

Phenological stage/standards	**Chemical/Fertilizer**	**% of inhibition peroxidation**			
		Concentration ($\mu g.mL^{-1}$)			
		125	250	500	1000
Standards	Ascorbic acid	-6.21	-15.99	-31.13	-39.29
	BHT	50.91	57.67	62.27	69.89
	Trolox	58.19	60.27	60.84	61.65
Vegetative stage	Control	14.36	20.57	25.69	31.28
	Chemical fertilizer	4.20	4.28	13.59	20.06
	Organic fertilizer	5.30	9.30	13.10	17.81
Reproductive stage	Control	8.25	11.46	17.02	21.74
	Chemical fertilizer	8.40	14.25	18.81	25.55
	Organic fertilizer	1.74	6.40	9.57	15.31

Table 7 Frequency of micronucleated polychromatic erytrocytes (MNPCE) in 1000 PCE and ratio between the number of polycromatic and normocromatic erythrocytes, by the formula PCE/(PCE + NCE), following the protocol of mutagenicity with hydroalcoholic extract of *Bidens pilosa* collected from three growth conditions of growth: absence of fertilizer (control), presence of bovine fertilizer (organic) and presence of chemical fertilizer (NPK), in reproductive stage.

Treatment	Growth conditions											
	Control				Organic fertilizer				Chemical fertilizer			
	MNPCE/1000 PCE ± SE		Ratio (PCE:PCE + NCE) ± SE		MNPCE/1000 PCE ± SE		Ratio (PCE:PCE + NCE) ± SE		MNPCE/1000 PCE ± SE		Ratio (PCE:PCE + NCE) ± SE	
NaCl 0.9%[1]	0,75	± 0,96 [a]	0,58	± 0,024[a]	0,75	± 0,96 [c]	0,58	± 0,024[a]	0,75	± 0,96 [bc]	0,58	± 0,024[a]
Cyclophosphamide[2]	56,08	± 6.66 [b]	0,44	± 0,041[c]	56,08	± 6.66 [a]	0,44	± 0,041[c]	56,08	± 6.66 [a]	0,44	± 0,041[b]
HAE 100mg.kg^{-1} b.w	3.08	± 1.67[a]	0.55	± 0.026[ab]	4.16	± 1.46[bc]	0.52	± 0.022[b]	4.08	± 2.31[b]	0.55	± 0.016[a]
HAE 200mg.kg^{-1} b.w	1.59	± 1.08[a]	0.55	± 0.027[ab]	5.33	± 2.22[b]	0.54	± 0.024[b]	1.58	± 1.16[bc]	0.56	± 0.027[a]
HAE 300mg.kg^{-1} b.w	1.58	± 0.99[a]	0.54	± 0.017[b]	4.58	± 2.77[bc]	0.55	± 0.020[b]	0.25	± 0.45[c]	0.56	± 0.021[a]

Equal letters represent statistical similarity (ANOVA, Tukey test, $P < 0.05$). SE = standard error. [1]negative control; [2]positive control.

4 Discussion

The application of mineral fertilizer (NPK) and organic fertilizer (bovine manure) increased the plant growth in *B. pilosa* over control (no fertilizer) in all growth parameters evaluated. The organic fertilizer presented better performance in comparison with mineral fertilizer for the leaf area and height, in reproductive and vegetative stages, respectively. Some research has revealed that the use of organic fertilizers increase the parameters of vegetative and reproductive growth [45, 46, 47, 48, 49]. Indeed, the mineral elements and harvest season is the one of the main factors influencing plant growth and development [4].

The increase of biomass is not always accompanied by the increase of secondary metabolites. The qualitative chemical analysis of hydroalcoholic extract of *B.* pilosa, from all treatments and phenological stages, revealed the presence of flavonoids, coumarins, steroids and naphthoquinones. In quantitative analyses, the HAE of control obtained higher tannins, phenols and flavonoids contents, in both harvesting stages, showing inversely proportional to biomass.

Studies have shown that nutrient poor soils have a lower growth rate and higher biosynthesis of all classes of secondary metabolites, except nitrogen compounds [8]. Nutritional stress has a marked effect on the level of secondary metabolites, especially phenolic compounds. Researches has shown that deficiency of nitrogen (N), phosphate (PO4), potassium (K), sulfur (S), iron (Fe) and magnesium (Mg) can increase the concentration of this compounds in different plant species. The higher accumulation of secondary metabolites occurs more frequently in plants subject to stress [50, 51, 52, 18]. Moreover, the content of tannins, phenols and flavonoids has varied significantly between phenological stages analyzed, which corroborates with others researchers who suggested that phenolic compounds vary significantly during life stages of the plants [13].

The different forms of plant cultivation and the phenological stages reflected in phytochemical changes, which resulted in significant differences in the antioxidant activity of HAE. To evaluate the antioxidant potential of a substance, it is necessary to combine different methodologies, since, due to the complexity of the oxidation-antioxidation process and owing to the chemical variety of antioxidant

components in the crude extract, no single method is able to provide a comprehensive picture of the antioxidant profile of a given sample [53, 54].

Antioxidants with free radical scavenging activities may have great relevance in the prevention and treatment of diseases induced by them, since free radicals are involved in the propagation of cellular damage [55]. This study evaluated the free-radical-scavenging capacity of the HAE using DPPH and ABTS assays. Both tests are colorimetric, easily reproducible and fast to perform [56, 53]. The results showed that the HAE from control exhibited better antioxidant activity by this mechanism in both phenological stages (vegetative and reproductive) (Table 4), when compared with the HAE from fertilizers. The scavenging activity improved at reproductive stage for all treatments and strong correlation was observed between TTC, TPC and TFC and this mechanism of action (Table 5), except TPC and TFC for DPPH at the reproductive stage, which showed low or weak correlation.

Thus, the results suggest that the phenolic compounds present in the extracts are important but not the only factor affecting antioxidant activity, as also reported by other authors [57]. These variations can be attributed to qualitative aspects, such as structural factors of individual antioxidants. In general, phenolics compounds have hydroxyl groups, which are good hydrogen-donors for neutralization of free-radicals [58]. The presence of other functional groups, such as double bond conjugated to phenolic group, the degree of hydroxylation and/or methoxylation and others groups, can play different functions and which are directly related to the different biological activities that such compounds may present (57, 59, 60, 61]. ABTS assay was more sensitive in the detection of antioxidant activity than the DPPH assay. This can be attributed to the higher reaction kinetics of ABTS and the lower capacity of DPPH to detect the antioxidant activity of compounds with higher polarity [62].

The lipid peroxidation inhibitory activity of the HAE was assessed by the β-carotene/linoleic acid system. This assay is based on the oxidation of linoleic acid due to the temperature, generating peroxyl free radicals due to the abstraction of hydrogen atom from carbon eleven (C_{11}) of linoleic acid. The radical will oxidize the highly unsaturated β-carotene, with loss of absorbance [11, 63). The presence of antioxidants in the extracts reduces the oxidation of β-carotene, so that the degradation rate of the β-

carotene depends on the antioxidant activity (63, 64]. The presence of antioxidant in HAE reduced the oxidation of β-carotene and this capacity varied significantly as function of fertilizer. The worst result was found for organic fertilizer and this result can be associated with the lowest TTC, TPC and TFC that this extract presents.

The evaluation of chelating activity is also widely used since transition metals can act as mediators of Haber-Weiss and Fenton reactions, with consequent generation of reactive species and induction of cellular damage [65, 66, 67, 68, 69]. This method is based in the formation of a stable complex of Ferrozine with Fe^{+2}. In presence of a chelating agent, the formation of this complex is reduced or prevented [41, 68]. The HAE from organic fertilizer it was greater for both phenological stages.

All antioxidant assays performed presented positive correlation of HAE with tannins content (Table 5). Researches demonstrate that tannins exert their antioxidant activity by scavenging free radicals, chelating trace metals and by binding proteins with suppression of their enzymatic activity [58]. This positive correlation was also observed by other researches [63, 70) for other plants. Furthermore, other researchers demonstrated that fertilizers influenced the phytochemical composition and the antioxidant activity of other species [71, 72].

The present study investigated the cytotoxic and mutagenic potential of *B. pilosa* HAE using the micronucleus test in bone marrow cells of Swiss mice, *in vivo*. The test was conducted only for the reproductive stage at three different concentrations (100, 200 and 300 mg.kg-1), because it is the stage most commonly used and did not exhibit any cytotoxic or mutagenic effects (Table 7), under these experimental conditions.

The micronucleus assay has been widely used to identify compounds that cause loss of whole chromosomes or chromosomal rupture, resulting from aneugenic and clastogenic events, respectively [73, 74, 75]. The mutagen cyclophosphamide was used as positive control. This chemical is a bifunctional alkylating agent, cytotoxic and mutagenic, that presents several mechanisms of action that cause damage to the genetic material observable microscopically (micronucleus) [76, 77, 78].

There is no agreement in the literature regarding the higher production of biomass and secondary metabolites in relation to the use of different types of fertilizers. The different species can respond in different ways to the same treatment, hindering a generalization. The cultivation of medicinal plants can facilitate the maintaining standards in quality, chemical compostition, growth and biological activities of the produce. The influence of fertilizer levels on growth and yield was reported for different species [79].

5. Conclusions

Under the present experimental conditions, our results showed that chemical and organic fertilizers increased all growth parameters analyzed, but the increase in biomass was not accompanied by an increase in the TTC, TPC and TFC. The effect of chemical and organic fertilizers was variable with respect to the antioxidant activity. In general, the HAE from control (absence of fertilizer) showed better antioxidant results. For the mutagenicity and cytotoxicity, the HAE from all treatments, (reproductive stage) was not mutagenic and cytotoxic. These results contribute to the safety assessment of HAE as medicinal plant for human use and for the standardization of its production evaluating the influence of fertilizers and harvest season on different parameters.

Conflict of interest: The authors declare no conflicts of interest.

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3.3 Manuscrito 3

O manuscrito intitulado "*Bidens pilosa* L. fractions from four populations: antioxidant activity by multiples assays and phytochemical analysis" será submetido para avaliação ao periódico *Food and Chemical Toxicology*.

Bidens pilosa L. fractions from four populations: antioxidant activity by multiples assays and phytochemical analysis

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Abstract

Bidens pilosa L., popularly known as "picão-preto", is widely distributed in the tropical and subtropical regions of the world, mainly in agricultural areas. Its aerial part is used in folk medicine for many applications. In this study, the extracts of the plants harvesting in four locations of Brazil and their fractions were obtained from a liquid-liquid partition using five solvents with crescent polarity (hexane, dichloromethane, ethyl acetate, butanol and water) resulting in five fractions. All fractions of each location were then analyzed for tannins, phenols and flavonoids contents and using a electrospray ionization negative FT-ICR mass spectrometry (ESI(-) FT-ICR MS). The phytochemical analysis revealed large qualitative and quantitative differences between the samples that reflected in the antioxidant activity. The antioxidant activity of these extracts was evaluated using four methods with different mechanisms: radical scavenging assays ($DPPH^{\bullet}$ and $ABTS^{\bullet+}$), chelating activity and inhibition of lipid peroxidation (β-carotene/linoleic acid system). High radical scavenging was found using the ethyl acetate fraction and this activity was strongly correlated with the phytochemical contents analyzed. The highest chelating activity was found using the aqueous fraction while the highest inhibition of lipid peroxidation was found in dichloromethane, aqueous and ethyl acetate fractions. The results indicated that *B. pilosa* fractions can be used potentially as a ready accessible and valuable bioactive source of natural antioxidant and highlight the importance of standardizing their growth condition and production of the extracts according to the objective.

Keywords: medicinal plants; *Bidens pilosa*; secondary metabolites; antioxidant activity.

1. Introduction

Oxidative stress represents a balance disturbance of the pro-oxidant and antioxidant reactions in biological systems, and produces free radicals as byproducts, especially reactive oxygen and nitrogen species (ROS and RNS) (Valko et al., 2007) that can attack various biomolecules including DNA, proteins and lipid, besides can related with the development of innumerous chronic diseases (Vasconcelos et al., 2007; Sharma et al., 2012).

Thus, the use of antioxidants for elimination of reactive species are especially important in impediment, prevention or removing damage induced by oxidative stress, directly or indirectly (Halliwell, 2007; Landete, 2013). Therefore, due to the possible adverse effects of synthetic antioxidants, food and pharmaceutical industries have turned their attention to natural antioxidants (Ebrahimabad et al., 2010; Tlili et al., 2014).

Previous studies have suggested that plant material, such as fruits, leaves and flowers, are potential sources of natural antioxidants such as phenolic compounds and other secondary metabolites (Jennings and Akoh, 2009; Trabelsi et al., 2013). However, the chemical compounds and the yield of plant extracts may vary according to a number of environmental factors, such as altitude, temperature and UV incidence (Köhlenon et al., 1999), and the solvent used for the extraction due to the polarity difference (Sasidharan et al., 2011).

Bidens pilosa L., commonly known as "picão-preto", is widely distributed in the tropical and subtropical regions of the world, mainly in agricultural areas, and has been traditionally used in foods and folk medicine for treatment of inflammation, jaundice, diabetes, cancer, hepatitis and many other disorders, without obvious adverse effects (Arthur et al., 2012). This specie is an outstanding source of natural compounds with more than 200 compounds identified, many of them related to their medicinal properties (Silva et al., 2011; Bairwa et al., 2010; Bartolome et al., 2013).

Researches has been reported the relationship between the therapeutic activities of *B. pilosa* and its antioxidant and anti-inflammatory capacity. Many studies have demonstrated that the aerial part of the plant has the ability to minimize lipid peroxidation (Ávila et al., 2015, Goudoum et al., 2016), act in the radical scavenging and chelate transition metals thus avoiding the formation of hydroxyl radicals (Kusano et al., 2003). Many biological and pharmacological assays have been reported its therapeutical activities: immunomodulatory (Horiuchi and Seyama, 2008), anthyperglycemic (Habeck, 2003), antimalarial (Oliveira et al., 2004; Kumari et al., 2009), antimicrobial (Silva Junior et al., 2014), hepatopretective (Suzigan et al., 2009), cytotoxic against several cell lines (Sundararajan et al., 2006; Kviecinski et al., 2008; Tagami et al., 2009; Abdou et al., 2010; Kumari et al., 2009), among others. However, studies that comparing phytochemical and antioxidant differences of the extracts obtained from different solvents and locations of harvest are insufficient and inconclusive. The elucidation of these factors is the key step in quality assurance and the standardization of phytopharmaceutical preparation of the plant.

Thus, the aims of the current study were to evaluate bioactive properties of fractions from *B. pilosa* hydroalcoholic extracts from four different locations of harvest by determining total tannins, phenols and flavonoids contents, and their antioxidant activity by multiples mechanisms of action. In addition, Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS), a valuable tool for the characterization and identification of compounds or functional groups, was used to identify some chemical constituents in the fractions of the different populations.

2 Materials and methods

2.1 Plant material and preparation of hydroalcoholic extract and fractions

Bidens pilosa L. were collected in four locations of Brazil: Afonso Cláudio (AC) (41º 09′ 58.57″ W; 20º 15′ 07.33″S), Barra de São Francisco (BSF) (40º 54′ 51.9″ W; 18º 44′ 43.9″ S), Cariacica (CA) (40º 23' 54.0" W; 20º 17' 28.5" S) and Muniz Freire (MF) (41º 25′ 22.593″ W; 20º 31′ 38.1008″ S), during January 2014. The aerial part of the plant was air-dried at room temperature and then ground for further analysis. Vouchers specimens were identified by Dra. Luciana Dias Thomaz from VIES herbarium of the Universidade Federal do Espírito Santo. The dried material was triturated and macerated with 70% ethanol (v/v), using a proportion of 1:5 for powder to solvent (w/v) for 72 hours. This process was repeated three times with the same powder. The resulting extracts were filtered and concentrated under vacuum rotary evaporator to remove the solvent and obtain the crude hydroalcoholic extracts of the *B. pilosa* (HAE).

Fractions were prepared from the hydroalcoholic extracts. Part of each HAE was dissolved in distilled water (100mL) for fractionation. The aqueous solution was fractionated with different solvents applying the liquid-liquid partition method with increasingly polar solvents (hexane, dichloromethane, ethyl acetate and butanol) and glass separatory funnel (Dorman and Hiltunen, 2004). The solvents of each fraction and the final aqueous fraction were evaporated in vacuum rotary evaporator. Five fractions were obtained by successive partition and designated as follows: hexanic (HF), dichloromethane (DCM), ethyl acetate (AcOEt), butanol (ButOH) and aqueous (WF). The yield of each fraction for each locality was calculated based on the amount of HAE used.

2.2 Phytochemical analysis

2.2.1 Phytochemical contents

The Folin-Ciocalteu method, as described by Zhang et al. (2006), was used for the determination of total phenolic content (TPC) of fractions. 20 µL of ethanol solution of each fraction (500 µg.mL^{1}) were added to 100 µL of Folin-Ciocalteu diluted in distilled water (1:10). After 5 minutes, 80 µL of Na_2CO_3 (7.5%) and the absorbance was read at 750 nm in spectrophotometric microplate reader (Epoch Microplate Spectrophotometer – BioTek) after 1 h of incubation in the dark at room temperature. The TPC was reported as mg gallic acid equivalent per gram of dry weight (mgGAE.g^{-1} d.w).

Total tannins content (TTC) was measured by the Folin-Denis method (Ryu et al., 2016) with minor modifications. 400 µL of ethanol solution of each fraction (500 µg.mL^{1}) were added to an equal volume of Folin-Denis reagent. After 3 min, 400 µL of Na_2CO_3 (8%) were added and after an hour, the solution was centrifuged at 200 rpm for 5 min and the absorbance measured at 725 nm. The TTC was reported as mg tannic acid equivalent per gram of dry weight (mgTAE.g^{-1} d.w).

The $AlCl_3$ method, as described by Dewanto et al. (2002) and Tlili et al. (2014), was used for the determination of total flavonoids content (TFC). 250 µL of methanolic solutions of the fractions (500 µg.mL^{1}) were mixed with 75 µL of $NaNO_2$ (7%) and 150 µL of $AlCl_3$ (10%). After 6 min, 500 µL of NaOH (1 M) was added and the absorbance was measured after 15 min of incubation in the dark, at room temperature. TFC was expressed as mg quercetin equivalent per gram of dry weight (mgQE.g^{-1} d.w). The analysis was performed in triplicate and conducted for the five fractions from all locations.

2.2.2 Mass spectrometry by ESI(-) FT-ICR MS analysis

The fractions of *Bidens pilosa* from all locations was analyzed in a mass spectrometer (Model 9.4 T Solarix, Bruker Daltonics, Bre-men, Germany), which was set to operate in negative ion

mode, ESI(-), over a mass range of m/z 200–1000. The fractions samples were diluted to 250 $\mu g.mL^{-1}$ in a 1:1 water-to-methanol ratio that contained 0.1% (m/v) of NH_4OH solution. The resulting solutions were analyzed in ESI (-) FT-ICR MS by direct infusion at a flow rate of 5 $\mu L.min^{-1}$ into the electrospray source in negative ion mode of acquisition (ESI(-)). The parameters of the ESI(-) source were as follows: nebulizer gas pressure of 1.0 bar, capillary voltage of 3.2 kV, and the capillary temperature of 250 °C. The ion accumulation time in the hexapolar collision cell was of 5.10^{-4}, followed by transport to the analyzer cell (ICR) through the multipole ion guide system. Each spectrum has been acquired by accumulation of 32 scans of the time-domain in 4 mega-point (Costa et al., 2015).

All FT-ICR MS data were externally calibrated using NaTFA solution (m/z 200 until 1200). A resolving power of m/Δm50% 500.000, in which Δm50% is the full peak with at a half-maximum peak height of m/z 428 and mass accuracy less than 1 ppm, provided unambiguous molecular formula assignments for the singly charged molecular ions. FT-ICR MS data were acquired and processed using the data analysis software (Bruker Daltonics, Bremen, Germany). The elemental compositions of the compounds were determined by measuring the *m/z* values. The degree of introduction for each molecule was determined from the value of DBE (*double bond equivalent*) (Destefani et al., 2014), following the equation: DBE = c – h/2 + n/2 + 1, where c, h, and n correspond to the numbers of carbon, hydrogen and nitrogen, respectively, in the given minimum formula from FT-ICR MS data. The proposed structures for formulas were assigned using the chemspider data base (www.chemspider.com).

2.3 Antioxidant activity assays

2.3.1 DPPH radical scavenging assay

The effect of the fractions and standards (Trolox and ascorbic acid) on DPPH radical was measured in spectrometric method modified for microplate reader. The samples (100 µL) were added to a methanolic solution (200 µL) of DPPH radical (0.3 mM). The mixture was shaken and the absorbance was measured at 517 nm after 30 min of incubation in the dark. The ability to scavenge the $DPPH^{\bullet}$ radical was calculated using the following equation: % inhibition of $DPPH^{\bullet}$ = [(Abso – Abs1) / Abso] x 100, where Abso = absorbance of control and Abs1 = absorbance of the sample. IC_{50} was calculated as the concentration of extracts required to scavenge 50% of DPPH radical.

2.3.2 $ABTS^{+\bullet}$ radical scavenging assay

The $ABTS^{+\bullet}$ scavenging activity of the fractions and standard Trolox were determined using a modified method as described previously by Re et al. (1999). The $ABTS^{+\bullet}$ was generated by a previous reaction with 5 mL of 7 mM ABTS solution and 88 µL of 140 mM potassium persulfate solution followed by incubation for 16 h in the dark, at room temperature. The resulting solution (work solution), blue-green, was adjusted to an absorbance of 0.70 ± 0.02, at 734 nm. 200 µL of work solution was added to 40 µL of ethanol solutions of each fractions/standard. The absorbance was measured at 734 nm, after 6 min of incubation at room temperature. The same formula used for $DPPH^{\bullet}$ assay was used for calculated the % inhibition of $ABTS^{+\bullet}$. Data were reported as an IC_{50} ($\mu g.mL^{-1}$).

2.3.3 Chelating activity on Fe^{+2} ions

The chelating activity assay was carried out according to the procedure of Tang et al. (2002). 22 µL of 2 mM $FeCl_2$ was mixed with 1 mL of methanolic solutions of samples and EDTA standard. Then, 43 µL of 5 mM ferrozine was added, mixed and the absorbance was measured

at 562 nm after 20 min. The percentage of inhibition of ferrozine-Fe^{+2} complex formation was calculate as following: Chelating activity (%) = (1 - Abs1/Abso) x 100, where Abso = absorbance of control and Abs1 = absorbance of the sample. The results were expressed IC_{50} ($\mu g.mL^{-1}$).

2.3.4 Linoleic acid peroxidation inhibition by β-carotene/linoleic acid system

This method is based on the discoloration of the β-carotene due to peroxides generated during the oxidation of linoleic acid at elevated temperature (Miller, 1971; Koleva et al., 2002; Hajlaoui, 2010). 80µL of linoleic acid and 530µL of Tween 40 were added to 1 mL of β-carotene dissolved in $CHCl_3$ ($0.5mg.mL^{-1}$). The chloroform was evaporated for 30 minutes with oxygenator. Then, 50 mL of oxygenated water was added, resulting in a reagent mixture. 250µL of the reagent mixture were mixed to 40µL of sample or standards (Trolox, ascorbic acid or BHT). The absorbance was measured at 470 nm immediately (t = 0 min) and after 120 min of incubation at 50ºC. The percentage of inhibition of peroxidation in term of β-carotene control was calculated using the following formula: $I(\%) = [(\Delta Abs_o - \Delta Abs_1)/\Delta Abs_o] \times 100$. The results were expressed as mM Trolox equivalent per gram of dry weight (mM $TE.g^{-1}$ d.w).

2.4 Statistical analysis

The results were expressed as the means ± standard error. For the phytochemical analysis and antioxidant activity, were performed ANOVA followed test-t ($P<0.05$) using ASSISTAT version 7.7 beta software (Assistat Software, Campinas, São Paulo, Brazil). Pearson correlations were performed by principal component analysis (PCA) using XLSTAT (2016) for Windows (Addinsoft, New York, USA), correlating phytochemical and antioxidant dates, in order to visualize relationships.

3. Results and Discussion

3.1 Fractions yields

The percentage yield of the different fractions of four *Bidens pilosa* populations is presented in Table 1. The results showed that the WF had the highest percentage yield, followed by ButOH, for all populations. In general, the fraction that obtained the worst yield in each location was AcOEt.

3.2 Phytochemical analysis

The total content of phenols, tannins and flavonoids of fractions from 4 populations are presented in Table 1. In the present study, the TPC, TTC and TFC of the samples significantly varied according to the polarity of the solvent used for fractionation and population (Table 1, t-test $P<0.05$). The highest TPC, TTC and TFC were found in the AcOEt from Cariacica, with 306.38 mgGAE.g^{-1}, 305.98 mgTAE.g^{-1} and 1,139.85 mgQE.g^{-1}, respectively, followed by DCM also from Cariacica, with 177.51 mgGAE.g^{-1}, 147.44 mgTAE.g^{-1} and 1,348.77 mgQE.g^{-1}, respectively. In all populations analyzed, the lowest amount of total flavonoids was found in WF. Similar results were found by Lee et al. (2013), in which AcOEt fraction exhibited high TPC and TFC in *Bidens pilosa* flowers. In the same research, the HF was the least effective in extracting flavonoids.

In this study, ESI(-) FT-ICR MS data for *B. pilosa* fractions for the 4 populations were gathered (Figure 1-5). More than 200 peaks were detected for the fractions in the mass range from 200 to 1,000 Da, making the analyzes relatively complex. As shown in Table 3, fractions extracts of *B. pilosa* by ESI(-)FT-ICR MS, 11 compounds were identified as fatty acids, phenolics, phenylpropanoids and flavonoids. Were detected fatty acids, such as α-

linolenic acid, 277.21748 m/z, and linoleic acid, 279.23312 m/z; phenylpropanoids, such as 1-O-caffeoyl-β-xylose, 311.07747 m/z and 3,4-dicaffeoylquinic acid, 515.11950, phenolics, such as Chlorogenic acid, 353.08809 m/z and foliachinenoside G, 375.20283 m/z and flavonoids, such as astragalin, 447.09383 m/z and quercetin-3-O-β-D-glucuronopyranoside, 477.06799 m/z. These results corroborate previous data demonstrating that hexane removes mainly fatty acids, such α-linolenic and linoleic acids (Fig 2), ethyl acetate and butanol removes mainly phenolic compounds, such as chlorogenic acid, 1-O-caffeoyl-β-xylose, 3,4-dicaffeoylquinic acid, quercetin-3-O-β-D-glucuronopyranoside and astragalin (Fig 1 and 4) (Simões et al., 2000). Moreira et al. (2014) demonstrated, similar to our study, that ethyl acetate and dichloromethane solvents were the best solvents for chlorogenic acid extraction, in studies with *Coffea Arabica*. However, our results demonstrate that butanol is also a good chlorogenic acid exchanger. This solvent was not tested by these authors.

Generally, the fractions had noticeable differences. Differences were also observed among populations. No fraction obtained from BSF plants had α-linolenic acid, 1-O-caffeoyl-β-xylose and (-)-3,5-dicaffeoylquinic acid, while only this location had linoleic acid and astragalin. Quercetin-3-O-β-D-glucuronopyranoside and methyl 3,5-di-O-caffeoyl quinate were identified only in Cariacica, while in this location it was not observed foliachinenoside G and 1,6-Bis-O-[(2E)-3-(4-hydroxyphenyl)-2-propenoyl]-β-D-glucopyranose, as in other locations studied. In all samples were observed chlorogenic acid and 3,4-dicaffeoylquinic acid, in which they can be suggested as a chemical marker of the plant. The ultra-high accuracy mass (error < 1 ppm) provided from the ESI(-) FT-ICR MS enabled us to propose a structural assignment using a database chemspider (www.chemspider.com) as displayed in Figure 6, but it was not possible to differentiate among the possible constitutional isomers.

The ESI(-) FT-ICR MS is an accurate tool for the characterization and identification of compounds present in an unknown mixture of plant extract (Eberhardt et al., 2007; Hazra et

al., 2007), where the spectrum of an unknown compound can be identified by comparison to a library of known compounds. In our study, this technique revealed that the phytochemical composition of the extract is variable according to the locations and the fraction. These differences are more perceptible when the compounds are displayed in graphical (Fig. 1-5).

The differences observed in yield, phytochemical content and phytochemical compounds can be explained by the environmental differences in the harvesting locations (AC, BSF, CA and MF) and the solvents used for the fractionation process. It is known that the yield and chemical composition of the extract from plant material is dependent on several variables, such as environmental conditions, genetic factors and the type and polarity of solvent used (Peschel et al., 2006; Barbosa-Pereira et al., 2013; Kaewseejan and Siriamornpun, 2015).

Extraction is the first step in the analysis of bioactive chemical compounds of medicinal plants, especially phenolic compounds, since there are many techniques and process, the chemical components can be variables (Sasidharan et al., 2011). Aqueous and butanol fractions for example, presented compounds with highly polar and polar components, while hexane and ethyl acetate fractions presented nonpolar and moderately polar components (Khoudja et al., 2014). Due to the combination of various types of bioactive compounds and phytochemicals with variable polarity, the identification and characterization of bioactive compounds remains a big challenge (Sasidharan et al., 2011).

Since the chemical composition of plants is extremely complex, it can occur concomitant extraction of various types of substances, pharmacologically active or not. Thus, solvent selection is important and depends on the specific nature of the bioactive compounds of interest. For the hydrophilic compounds extraction it is used polar solvents such as ethyl acetate while for extraction of lipophilic compounds other solvents, such as dichloromethane and butanol, are used (Costa, 2000; Cosa et al., 2006).

In addition, due to the inability to move, plants have developed mechanisms to cope with unfavorable environmental conditions. One of those mechanisms is the synthesis of secondary metabolites that represents a chemical interface between the plants and the surrounding environment. Thus, the variation of the composition and concentration of secondary plant metabolites are strongly dependent on the growth conditions that, by different mechanisms, can alter the gene expression of the metabolic pathways responsible for the production and accumulation of the related compounds (Gobbo-Neto and Lopes, 2007; Ramakrishna and Ravishankar, 2011). Research's demonstrate that the levels of flavonoids, for example, increase in response to various factors, such as strong light, ultraviolet (UV) radiation, low/high temperature, heavy metals, drought, etc., being all stress conditions a source of free radicals for the plant (Mierziak, Kostyn and Kulma, 2014). In our study, the locations variated considerately in altitude (AF: 768 m, BSF: 233 m, CA: 12 m and MF: 522 m) and in UV radiation incidence (AF: 180-200, BSF: 220-240, CA: 120-100 and MF: 160-180). In addition, significant nutritional differences were observed in the soil, as demonstrated in Table 2. In CA soil, for example, was detected high content of heavy metals, such as iron and copper, which may have contributed to the greater synthesis of flavonoids. In addition, other not controlled factors such as herbivory and the microorganisms present on site, may have affected positively or negatively the phytochemical contents/composition (Michalak, 2006; Nasin and Dhir, 2009).

3.3 Antioxidant activity

Due to the presence of different antioxidant components and owing to the complexity of the oxidation-antioxidation processes (Khoudja et al., 2014), different antioxidant trials were performed to providing a comprehensive profile of the samples by four *in vitro* methods: free

radical scavenging activities (by $DPPH^{\bullet}$ and $ABTS^{+\bullet}$ methods), chelating activity on Fe^{+2} ions and linoleic acid peroxidation inhibition by β-carotene/linoleic acid system.

The fractions from all locations showed concentration-dependent antioxidant activity in the free radical scavenging ($DPPH^{\bullet}$ and $ABTS^{+\bullet}$) and chelating activities (data not shown). The results were reported as IC_{50} value in which lower IC_{50} corresponds to a higher antioxidant activity (Table 4). The $DPPH^{\bullet}$ and $ABTS^{+\bullet}$ assays are widely used to evaluate reducing substances and to investigate the activity of free radical scavenging mainly by donating a hydrogen atom (Souza et al., 2007; Kaewseejan and Siramornpun, 2015). Both tests showed the highest scavenging activity value in AcOEt, followed by ButOH fractions for all locations (Table 3). Our results it was in agreement with that of Lee et al (2013) that showed AcOEt as the better fraction for this antioxidant mechanism.

The DPPH● and ABTS+● scavenging activity showed the highest value in Cariacica AcOEt fraction, with IC_{50}= 59.23 and IC_{50}= 15.48, respectively. These values were statistically equal and lower than the Trolox standard IC_{50}, that have IC_{50}= 55.14 and 29.34 respectively (Table 4). There are larger differences between the least effective fraction (WF) and the most effective fractions (AcOEt and ButOH), as shown in Table 4 (t-test, P<0.05). ABTS assay is more sensitive to identifying the antioxidant activity since it has capable to identify compounds with high polarity (insoluble in organic solvents) and has faster reaction kinetics (Lee et al., 2015). Consequently, this study shows that the ABTS assay presented lower IC_{50} values. This fact is evident in WF, in with no activity was detected in DPPH assay (Table 4) due to the inability of DPPH detect antioxidant activity of high polar compounds.

Since transition metals ions are involved in ROS formation by Fenton reactions, with possible generation of hydroxyl radicals ($OH^{\bullet}$) which are highly reactive and harmful to biomolecules (Birben et al., 2012; Lone et al., 2013; Llorent-Martinez et al., 2017), the chelating activity was evaluated. As shown in Table 4, the highest activity value was found in WF, followed by

ButOH fractions. The best fraction by free radical scavenging mechanism (AcOEt) was the worst by this mechanism. Fractions from MF obtained the lower IC_{50} values into each fraction, except in HF (Table 4). These data can be attributed to the structural factors of the individual antioxidants (Erkan et al., 2008; Lu et al., 2014). Some flavonoids, for example, are able to chelate metals, such as Fe^{+2}, by bind to catechol group localized within the B ring, to the 3-hydroxyl and 4-oxo group of the heterocyclic ring and to the 4-oxo and 5-hydroxyl group between the heterocyclic and A rings (Mierziak, Kostyn, and Kulma, 2014), minimizing damages to many molecules and cell structures (Valko et al., 2007).

The inhibition of lipid peroxidation of fractions as also shown in Table 4, and compared to BHT and Trolox standards. Among all the fractions, WF showed the strongest activity, followed by DCM fractions, while ButOH exhibited the lowest activity. The location with higher TEAC value was Afonso Claudio, with TEAC= 0.449 and 0.459 mM TE.g-1, for WF and DCM fractions, respectively. It is known that lipid peroxidation is a dangerous process because it leads to a chain reaction with formation of different substances, among them the malondialdehyde (MDA), a carcinogenic and toxic product (Birben et al., 2012, Valko et al., 2007). In this method are evaluates antioxidant substances that are capable of neutralizing the radicals generated during the peroxidation of linoleic acid and, consequently reduce the peroxidation of β-carotene (Lu et al., 2014).

3.4 Correlation analysis

The correlation coefficients (r) between the mean values obtained from each assay were analyzed by performing a Pearson test with the purpose evaluating the relations between the evaluated phytochemical contents (TTC, TPC and TFC) and the antioxidant activities. For the AcOEt fraction, the TTC, TPC and TFC were strongly correlated with scavenging assays

against $DPPH^{\bullet}$ (*r*= -0.996, *r*= -0.999 and *r*= -0.996, respectively) and $ABTS^{\bullet+}$ (*r*= -0.897, *r*= -0.936 and *r*= -0.896, respectively) and moderately with β-carotene assay (*r*= -0.774, -0.795 and -0.765) and chelating activity (*r*= 0.613, *r*= 0.641, 0.622). In DCM fraction, TTC, TPC and TFC were correlated with $DPPH^{\bullet}$ (*r*= -0.979, *r*= -0.888 and *r*= -0.981, respectively), $ABTS^{\bullet+}$ (*r*= -0.978, *r*= -0.881 and *r*= -0.986, respectively), chelating activity (*r*= 0.994, *r*= 0.932 and *r*= 0.964, respectively) and β-carotene assay (*r*= -0.778, *r*= -0.605 and *r*= -0.940, respectively).

In both fractions, it was observed that the high levels of TTC, TPC and TFC were accompanied by potent $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radical scavenger activities, suggesting that the polyphenols may be the principal constituents responsible for the antiradical properties. Our findings were in agreement with previous studies with *B. pilosa* and other species reported this strong positive correlation (Lee et al., 2013; Kaewseejan and Siriamornpun, 2015; Llorent-Martinez et al., 2017). In addition, the higher TTC, TPC and TFC were accompanied by reducing chelating ability for these fractions. This fact suggests that metal chelating activity depends not only on the content of phenolic, but also on the nature of these compounds, their structures, as well as the presence of other chelating agents, such as polysaccharides or peptides. Some authors reported that these molecules plays more effective metal chelation ability of ferrous ions than phenolic compounds (Rice-Evans et al., 1996; Wang; Jónsdóttir and Ólafsdóttir, 2009; Llorent-Martinez et al., 2017).

For HF, the TTC was strongly correlated with chelating and $DPPH^{\bullet}$ activities (*r*= -0.913 and *r*= -0.906, respectively) and TFC was moderately correlated with $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ (*r*= -0.730, *r*= -0.641, respectively). In ButOH, TTC and TPC were correlated with $ABTS^{\bullet+}$ (*r*= -0.648, *r*= -0.778, respectively) and β-carotene (*r*= -0.902, *r*= -0.875, respectively). Finally, for WF the TPC was correlated with $ABTS^{\bullet+}$, chelating activity and β-carotene (*r*= -0.910, *r*= -0.641 and *r*= -0.652, respectively). In this fraction, TTC and TFC were correlated only β-

carotene (r= -0.755, r= -0.940, respectively). The absence of correlations between some contents evaluated with the antioxidant assays revealed that other factors, as cited above, such as differences in structure, the type of substituent groups and number or position of OH groups, can be the responsible for the differences in the antioxidant activity observed. The differences in the chemical constituents between the fractions (Fig 6), revealed by FT-ICR MS analysis, can explain the differentiated performance of the samples in different mechanisms of antioxidant activity.

4. Conclusions

The results of this screening experiment demonstrated that the different locations of harvest the plant for the HAE extract production and the different solvents used for fractionation influenced quantitatively and qualitatively the phytochemical compounds of *Bidens pilosa*, reflecting differences in antioxidant activity. AcOEt had the highest TTC, TPC and TFC, and the exhibited the highest antioxidant activities, especially ABTS and DPPH. WF showed the strongest chelating activity, and DCM judiciously with WF and AcOEt exhibited good protection against lipid peroxidation. The correlation analyzes suggest that the molecular differences is one of the factors that can influenced the antioxidant activity. Therefore, the findings of this work are useful to further research such as the identification of specific compounds responsible for the antioxidant activities, and to demonstrate the importance of standardizing the growth conditions of the specie to obtain homogenous and quality samples.

5. Conflict of Interest

The authors declare that there are no conflicts of interest.

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Table 1. Total phenols, tannins and flavonoids contents of *Bidens pilosa* fractions extracts collected from four localities: Afonso Claudio, Barra de São Francisco, Cariacica and Muniz Freire.

Plant extract (fractions)	Localization	Yields (%)	TPC (mg GAE.g^{-1} ± SE)	TTC (mg TAE.g^{-1} ± SE)	TFC (mg QE. g^{-1} ± SE)
AcOEt	Afonso Cláudio	2.07	210,25^{b} ± 3,34	197,49^{b} ± 1,18	610,01^{b} ± 4,98
	BSF	0.59	162,71^{d} ± 4,71	163,51^{d} ± 1,30	443,75^{d} ± 2,26
	Cariacica	4.23	306,38^{a} ± 4,26	305,98^{a} ± 2,38	1139,85^{a} ± 9,99
	Muniz Freire	1.07	197,12^{c} ± 3,48	187,48^{c} ± 0,56	551,67^{c} ± 1,88
ButOH	Afonso Cláudio	5.85	33,88^{c} ± 0,59	166,80^{b} ± 5,28	380,39^{c} ± 7,68
	BSF	6.51	59,76^{b} ± 5,79	153,77^{b} ± 3,15	322,67^{d} ± 0,63
	Cariacica	11.12	87,71^{a} ± 4,12	37,10^{c} ± 1,87	559,19^{a} ± 4,61
	Muniz Freire	8.15	57,26^{bc} ± 12,50	181,82^{a} ± 2,19	436,85^{b} ± 3,76
DCM	Afonso Cláudio	0.79	133,69^{b} ± 2,71	111,48^{b} ± 0,75	487,67^{b} ± 12,81
	BSF	2.56	116,24^{c} ± 3,96	104,59^{c} ± 0,89	541,62^{b} ± 5,14
	Cariacica	4.54	177,51^{a} ± 1,13	147,44^{a} ± 0,11	1348,77^{a} ± 33,04
	Muniz Freire	7.15	30,79^{d} ± 0,44	77,25^{d} ± 1,14	328,628^{c} ± 11,53
HF	Afonso Cláudio	2.89	33,88^{c} ± 0,59	45,47^{a} ± 1,63	555,43^{ab} ± 6,05
	BSF	3.27	65,50^{ab} ± 1,32	28,22^{b} ± 1,42	566,72^{ab} ± 7,68
	Cariacica	10.14	87,71^{a} ± 4,12	39,80^{a} ± 4,83	584,92^{a} ± 10,88
	Muniz Freire	4.92	57,26^{bc} ± 12,50	21,48^{c} ± 0,45	533,79^{b} ± 9,99
WF	Afonso Cláudio	80.73	39,18^{c} ± 1,28	35,45^{b} ± 1,50	189,03^{a} ± 3,82
	BSF	85.68	51,38^{ab} ± 3,38	49,12^{a} ± 1,39	201,58^{a} ± 1,88
	Cariacica	58.75	64,03^{bc} ± 4,56	37,44^{b} ± 3,39	194,68^{a} ± 7,24
	Muniz Freire	71.103	70,94^{a} ± 7,06	45,49^{b} ± 1,01	194,68^{a} ± 1,66

AcOEt: ethyl acetate fraction; ButOH: butanol fraction; DCM: dichloromethane fraction; HF: hexane fraction; WF: aqueous fraction. All the values are expressed as mean ± SE (n=3); SE: standard error; TTC: Total tannins content; TPC: total phenols content; TFC: total flavonoids content. $^{a-d}$Means with same superscripts type indicated no significant difference, ANOVA, test-t ($p < 0.05$).

Table 2 Chemical characterization of soil samples. The soil composition analysis was conducted by the Agronomic Analysis Laboratory and Consulting FULLIN-LTDA (Linhares/ES, Brazil) for the 4 locations.

Characteristic	**AC**	**BSF**	**CA**	**MF**
Mehlich phosphorus[1] ($mg.dm^3$)	3	29	52	12
Potassium (K)[1] ($mg.dm^3$)	76	94	39	59
Sulfur (S)[2] ($mg.dm^3$)	14	13	18	11
Calcium (Ca)[3] (cmol)	1.3	5.9	5.0	4.9
Magnesium (Mg)[3] (cmol)	0.5	1.4	1.7	1.4
H+Al[4] (cmol)	2.8	1.8	2.5	1.7
pH in water[5]	5.7	6.6	6.1	6.3
Organic matter[8] ($dag.Kg^{-1}$)	1.4	4.6	3.3	3.5
Iron (Fe)[1] ($mg.dm^3$)	100	116	311	60
Zinc (Zn)[1] ($mg.dm^3$)	1.7	14.3	7.3	8.6
Copper (Cu)[1] ($mg.dm^3$)	1.0	0.6	1.9	0.8
Manganese (Mn)[1] ($mg.dm^3$)	26	56	48	271

AC: Afonso Claudio; BSF: Barra de São Francisco; CA: Cariacica; MF: Muniz Freire. [1]Extraction: HCI 0.05 mol/L+H_2SO_4 0.025 mol/L; [2]Extraction: $Ca(H_2PO_4)_2$ 0.01 mol/L; [3]Extraction: KCI 1mol/L; [4]Solution SMP buffer; [5]pH in H_2O 1:2.5; [6]Oxidation: $Na_2Cr_2O_7$ $2H_2O$ + 4 mol/L H_2SO_4 10 mol/L

Table 3 Compounds derivative of butanol, ethyl acetate, dichloromethane, hexane and aqueous fractions of *Bidens pilosa* from four locations, as identified from ESI(-)-FT-ICR MS.

Identification	Theoretical m/z	m/z measured	Identified compounds	Molecular formula	DBE	Error	Reference
1	277.21730	277.21748	α-Linolenic acid	$C_{18}H_{30}O_2$	4,5	-0,18	*
2	279.23295	279.23312	Linoleic acid	$C_{18}H_{32}O_2$	3,5	-0,60	*
3	311.07724	311.07747	1-O-caffeoyl-β-xylose	$C_{14}H_{15}O_8$	7.5	-0.75	*
4	353.08781	353.08809	Chlorogenic acid	$C_{16}H_{18}O_9$	8,5	-0.91	*
5	375.20244	375.20283	Foliachinenoside G	$C_{18}H_{32}O_8$	3,5	-1.04	*
6	447.09329	447.09383	Astragalin	$C_{21}H_{20}O_{11}$	12,5	-1,22	*
7	471.13017	471.12967	1,6-Bis-O-[(2E)-3-(4-hydroxyphenyl)-2-propenoyl]-β-D-glucopyranose	$C_{24}H_{24}O_{10}$	13,5	-1.07	*
8	477.06746	477.06799	Quercetin-3-O-β-D-glucuronopyranoside	$C_{21}H_{18}O_{13}$	13.5	-0,53	*
9	515.1199	515.11950	3,4-Dicaffeoylquinic acid	$C_{25}H_{24}O_{12}$	14,5	-0.77	*
10	529.13515	529.13565	Methyl 3,5-di-O-caffeoyl quinate	$C_{26}H_{26}O_{12}$	14,5	-1.08	*
11		551.09701	(-)-3,5-Dicaffeoylquinic acid	$C_{25}H_{24}ClO_{12}$	13,5	-0,83	*

*Silva et al., (2011).

Table 3. Antioxidant activities of *Bidens pilosa* fractions obtained from plants of four populations: Afonso Claudio, Barra de São Francisco, Cariacica and Muniz Freire.

Plant extract/ chemical	Localization	DPPH	ABTS	Chelating activity	β-carotene linoleic acid
		IC_{50} (µg.mL^{-1})	IC_{50}, (µg.mL^{-1}±SE)	IC_{50} (µg.mL^{-1}±SE)	TEAC (mM TE.g^{-1}±SE)
Ascorbic acid	-	29.21^{a} ± 0.34	-	-	n.d*
Trolox	-	55.14^{b} ± 0.51	29.34^{a} ± 0.60	-	-
BHT	-	-	-	-	2,197
EDTA	-	-	-	17,67^{a} ± 0,25	-
AcOEt	Afonso Cláudio	104,84^{c} ± 1,08	17,57^{cd} ± 0,05	4866,73^{b} ± 171,3	0,273
	BSF	127,16^{d} ± 1,30	21,54^{b} ± 2,24	2127,57^{c} ± 68,5	0,289
	Cariacica	59,23^{b} ± 0,36	15,48^{d} ± 0,17	4617,03^{b} ± 191,4	0,222
	Muniz Freire	113,57^{e} ± 1,58	19,31^{bc} ± 0,69	1298,58^{d} ± 77,8	0,237
ButOH	Afonso Cláudio	138,19^{c} ± 4,35	37,28^{b} ± 1,29	773,64^{b} ± 3,15	n.d
	BSF	264,59^{d} ± 5,75	35,48^{b} ± 1,18	1759,68^{c} ± 9,12	0,153
	Cariacica	145,34^{c} ± 1,07	35,56^{b} ± 1,01	954,89^{d} ± 2,05	0,268
	Muniz Freire	128,15^{e} ± 2,03	36,99^{b} ± 0,79	742,35^{e} ± 1,59	n.d
DCM	Afonso Cláudio	237,13^{c} ± 4,16	36,99^{b} ± 0,61	1631,70^{b} ± 71,61	0,459
	BSF	255,36^{c} ± 16,56	38,71^{b} ± 0,50	1599,66^{b} ± 130,37	0,396
	Cariacica	124,79^{d} ± 4,06	18,88^{c} ± 0,98	3877,42^{c} ± 83,41	0,282
	Muniz Freire	294,94^{e} ± 14,34	45,42^{d} ± 0,38	184,52^{d} ± 4,43	0,428
HF	Afonso Cláudio	1259,13^{c} ± 11,11	227,80^{bc} ± 2,58	751,47^{b} ± 4,08	0,185
	BSF	1411,27^{d} ± 62,11	213,34^{c} ± 2,24	1694,74^{c} ± 71,66	0,356
	Cariacica	1332,39^{dc} ± 32,47	240,72^{b} ± 0,17	1141,41^{d} ± 41,04	0,201
	Muniz Freire	1749,26^{e} ± 63,32	283,17^{d} ± 0,69	1558,20^{c} ± 53,42	0,218
WF	Afonso Cláudio	n.d	352,71^{b} ± 7,54	162,30^{b} ± 1,09	0,449
	BSF	n.d	358,69^{b} ± 11,82	164,01^{bc} ± 0,54	0,188
	Cariacica	n.d	212,48^{c} ± 7,72	165,89^{c} ± 0,44	0,242
	Muniz Freire	n.d	84,15^{d} ± 5,51	120,50^{d} ± 0,20	0,253

AcOEt: ethyl acetate fraction; ButOH: butanol fraction; DCM: dichloromethane fraction; HF: hexane fraction; WF: aqueous fraction. All the values are expressed as mean ± SE (n=3); SE: standard error; $^{a\text{-}e}$Means with same superscripts type indicated no significant difference into the same fraction, ANOVA, test-t ($p < 0.05$; n.d, not detected value; *Prooxidant activity.

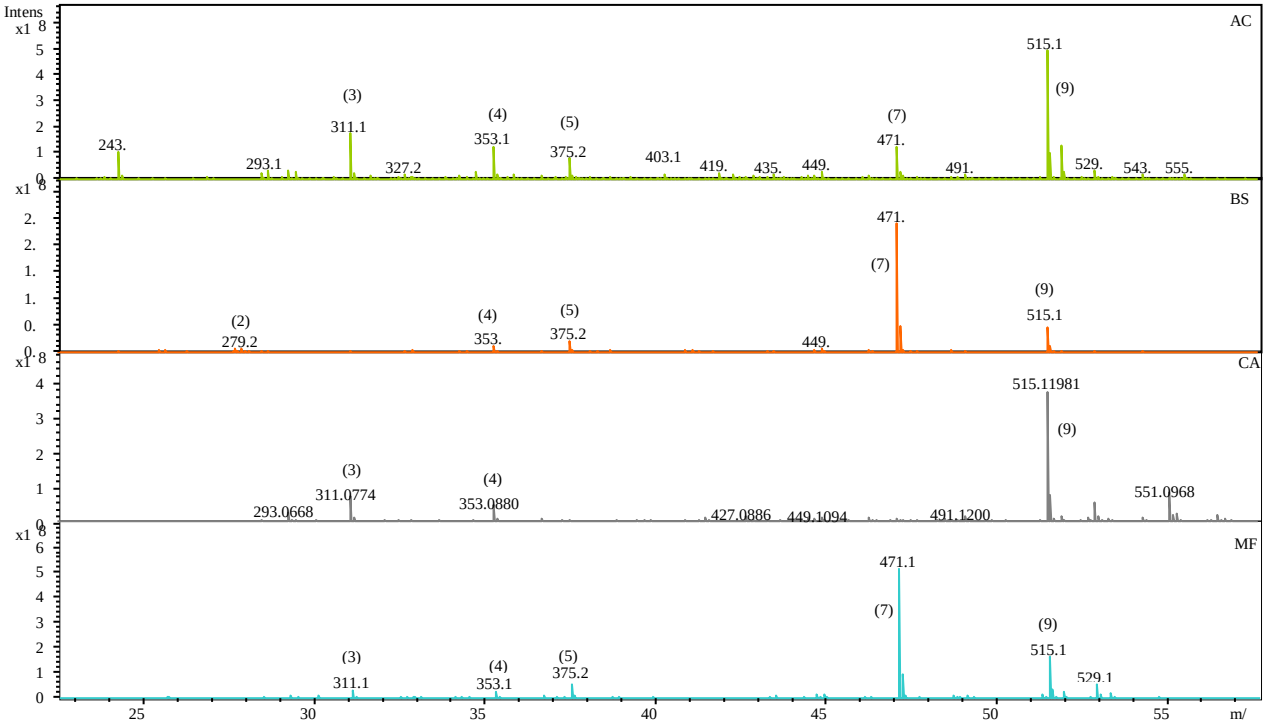


Figure 1. ESI(-)FT-ICR mass spectrum of ethyl acetate fractions of *Bidens pilosa* L. extracts from four locations: Afonso Claudio (AC), Barra de São Francisco (BSF), Cariacica (CA) and Muniz Freire (M).

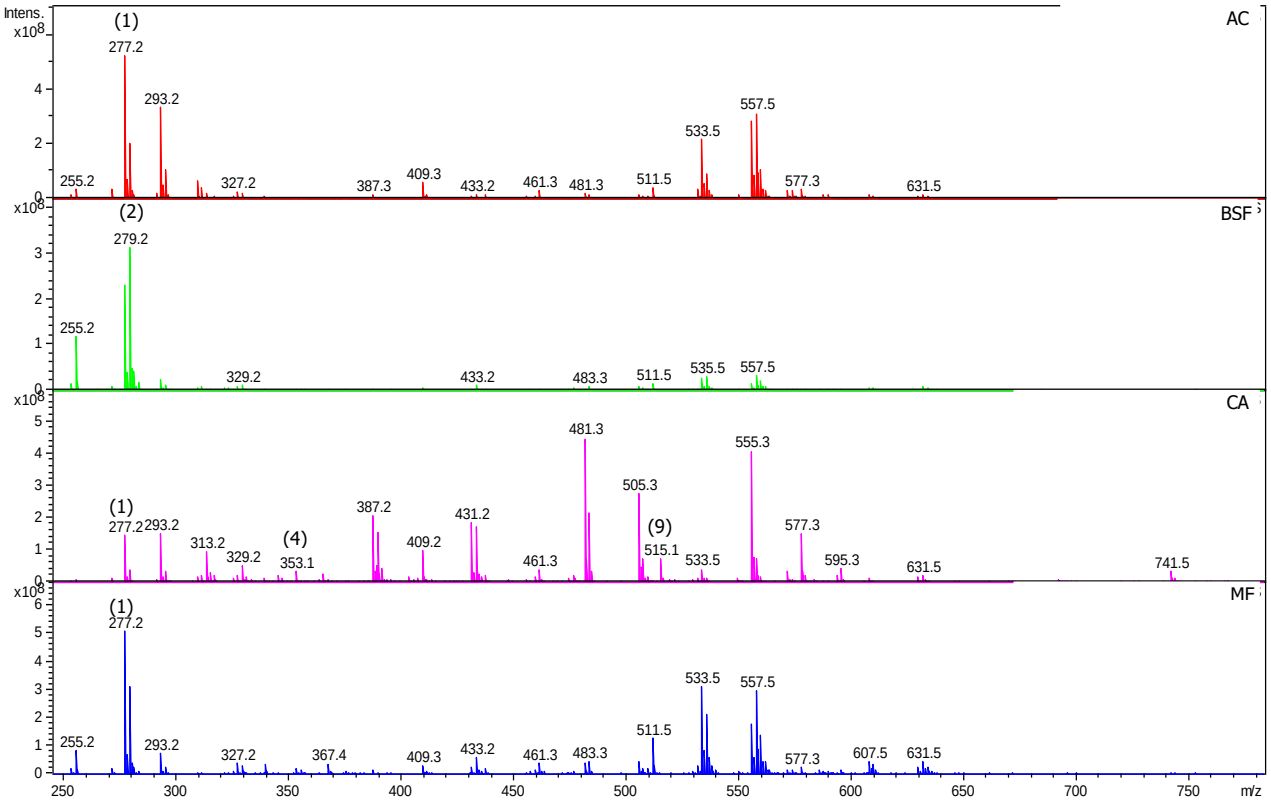


Figure 2. ESI(-)FT-ICR mass spectrum of hexane fractions of *Bidens pilosa* L. extracts from four locations: Afonso Claudio (AC), Barra de São Francisco (BSF), Cariacica (CA) and Muniz Freire (M).

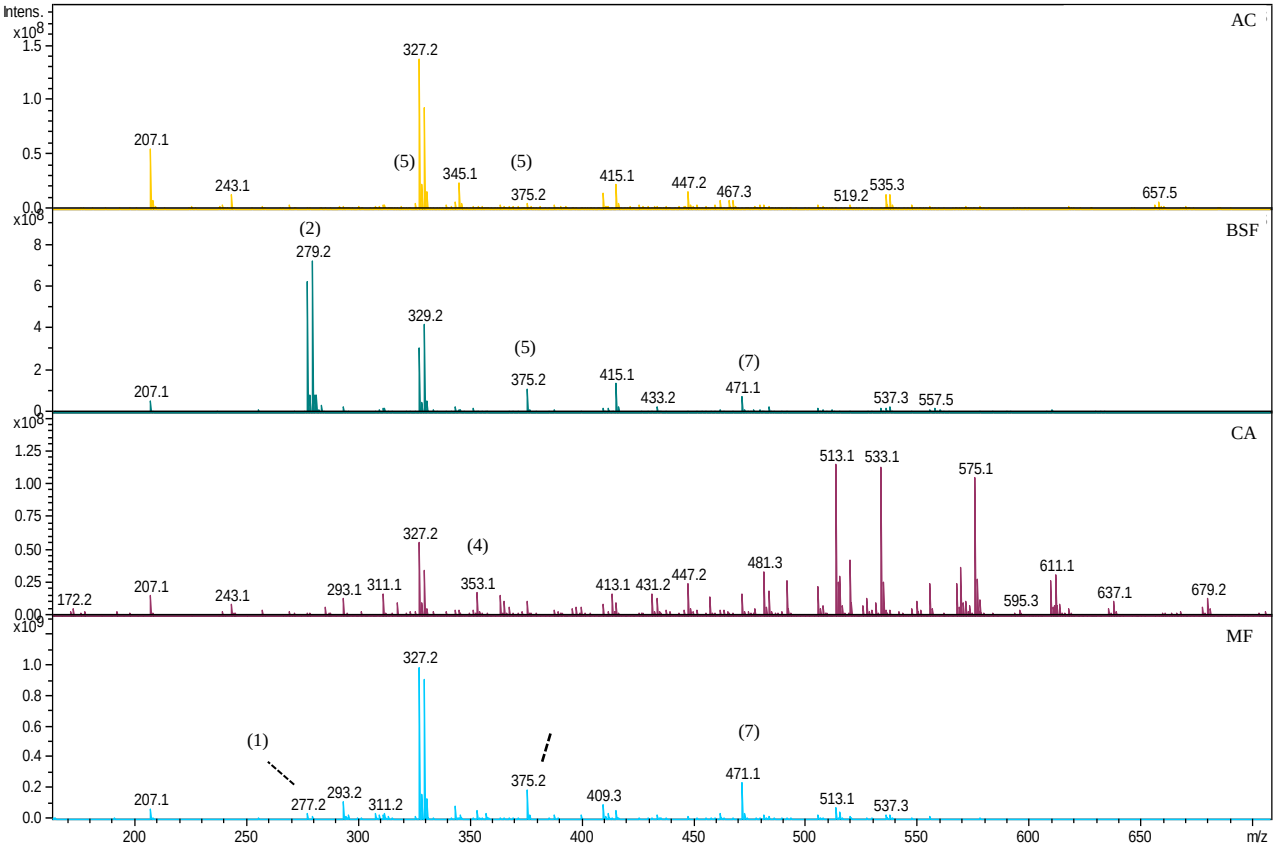


Figure 3. ESI(-)FT-ICR mass spectrum of dichloromethane fractions of *Bidens pilosa* L. extracts from four locations: Afonso Claudio (AC), Barra de São Francisco (BSF), Cariacica (CA) and Muniz Freire (MF).

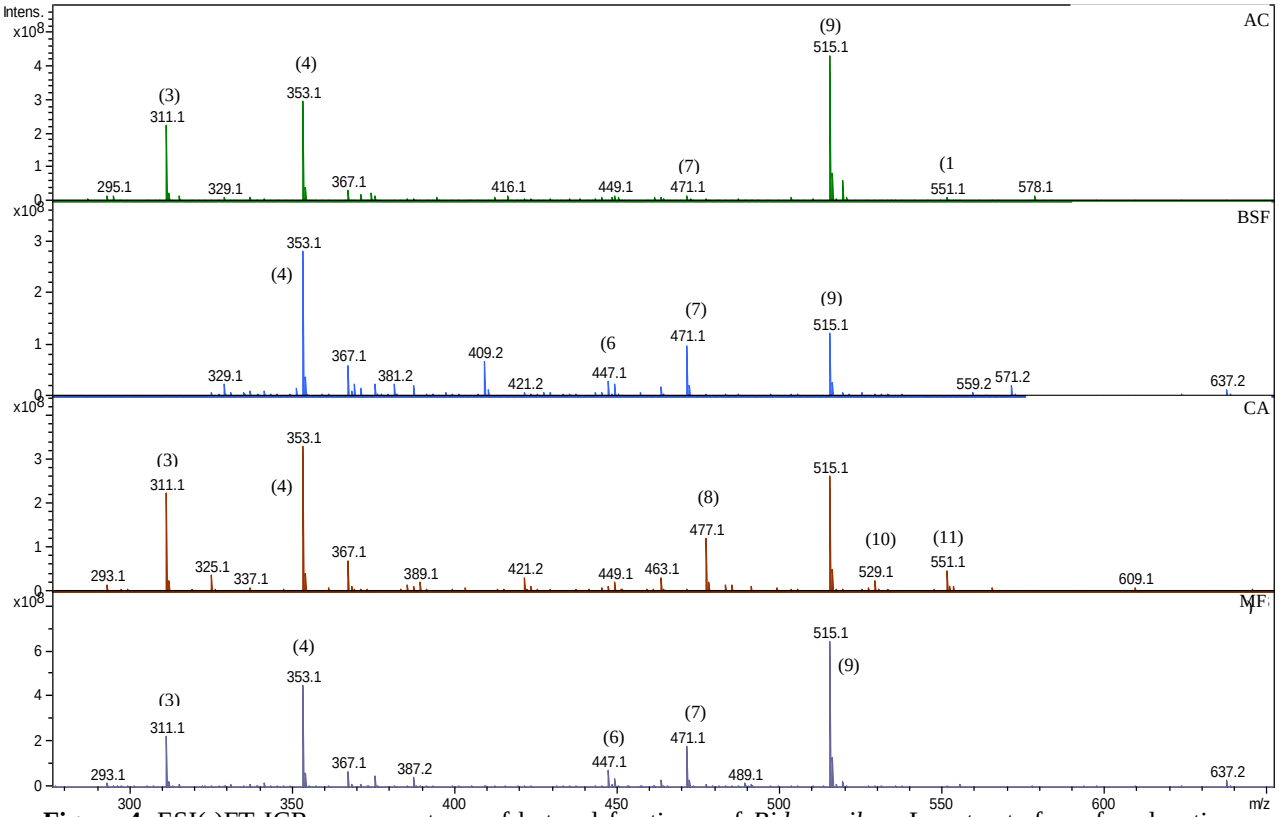


Figure 4. ESI(-)FT-ICR mass spectrum of butanol fractions of *Bidens pilosa* L. extracts from four locations: Afonso Claudio (AC), Barra de São Francisco (BSF), Cariacica (CA) and Muniz Freire (MF).

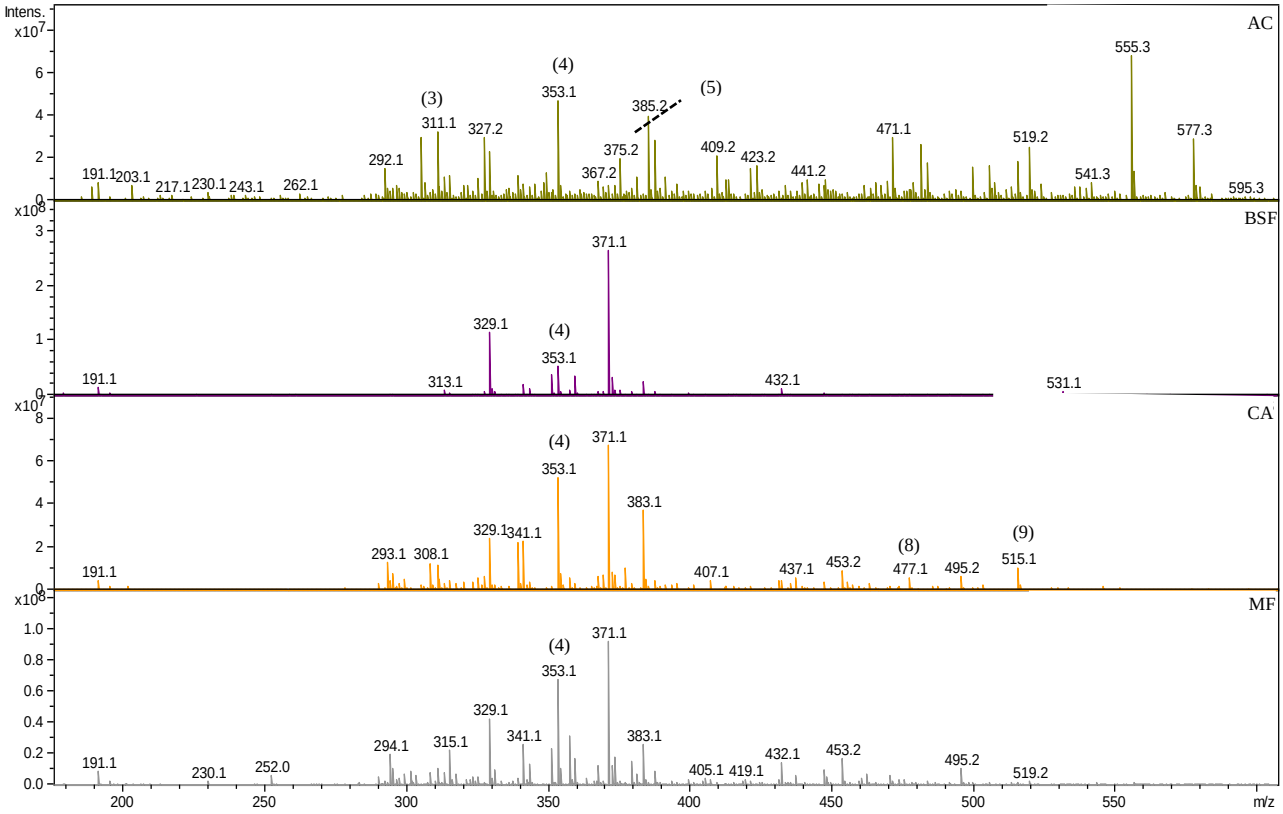
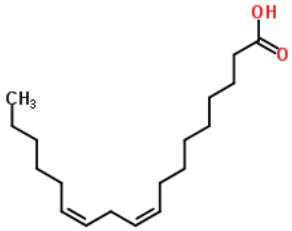
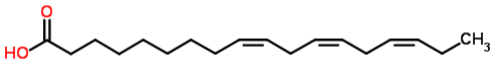


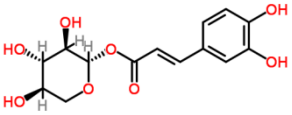
Figure 5. ESI(-)FT-ICR mass spectrum of aqueous fractions of *Bidens pilosa* L. extracts from four locations: Afonso Claudio (AC), Barra de São Francisco (BSF), Cariacica (CA) and Muniz Freire (MF).



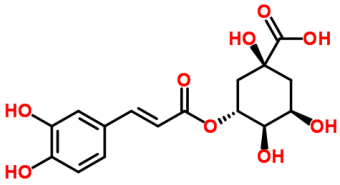
Linoleic acid



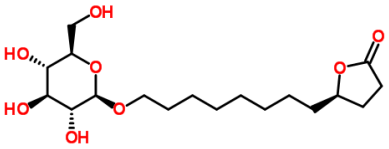
α-Linolenic acid



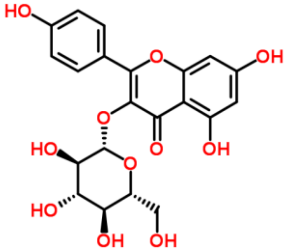
1-O-caffeoyl-β-xylose



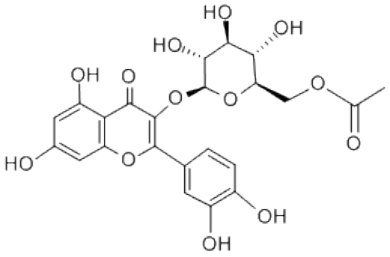
Chlorogenic acid



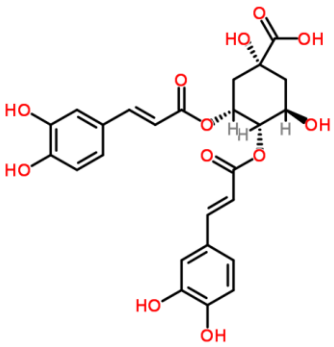
Foliachinenoside G



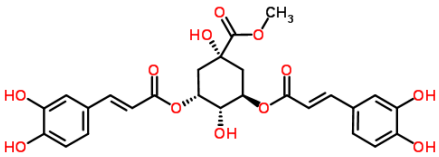
Astragalin



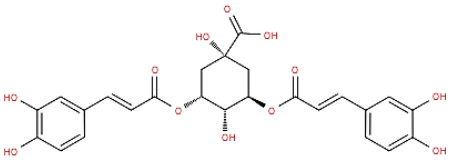
Quercetin-3-O-β-D-glucuronopyranoside



3,4-Dicaffeoylquinic acid



Methyl 3,5-di-O-caffeoyl quinate



(-)-3,5-Dicaffeoylquinic acid

Figure 6. Chemical structures identified from *Bidens pilosa* L. fractions from four localities.

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